The Cellular Response to Combined Treatment with the Microtubule Stabilizing Agent Patupilone and Ionizing Radiation: Regulation of Matrix Metalloproteinase Activity

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Abstract

The combined application of the novel, clinically relevant microtubule stabilizing agent (MTSA) patupilone and ionizing radiation (IR) is a very promising treatment modality in cancer therapy as previously investigated at a preclinical level in our laboratory. Patupilone (epothilone B) is a compound of bacterial origin, which binds to the same site on beta-tubulin as taxanes, but has number of advantages over the later, e.g. potent cytotoxicity in multi-drug resistant tumors and – due to its relatively simple chemical structure – the availability of derivatives with improved pharmacological properties. Patupilone has a strong supra-additive inhibitory effect on the growth of tumor xenografts in mice when applied together with IR. Interestingly, the additive cytotoxic effect was much less pronounced *in vitro* indicating that an additional effect on the level of tumor microenvironment must exist. While the initial hypothesis was that patupilone targets neovascular endothelial cells directly, further investigations revealed that the additive effect *in vivo* could only be observed in tumors xenografts derived from patupilone sensitive cancer cells. Thus the effect on the tumor microenvironment might be induced in an indirect way, mediated by tumor cells, for example via the secretion of biologically active molecules.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases with a broad variety of substrates including components of the extracellular matrix and growth factors (e.g. the transforming growth factor β (TGF- β). MMPs play an important role in the regulation of cell survival, apoptosis, angiogenesis, matrix remodelling and metastasis. The function of MMPs is strictly regulated by a complex activation processes (MMPs are expressed as zymogens) and natural inhibitors of their enzymatic activity. The tissue inhibitors of MMPs (TIMPs) represent a group of the most important players in the MMP regulation cascade currently known. TIMPs are a family of small proteins (size of 20-30 kDa), that contains 4 members: TIMP-1-4. All 4 TIMPs are potent inhibitors of the MMP enzymatic activity; TIMP-2 was also shown to be a co-activator of MMP-2 zymogen. Additionally, TIMPs have MMP independent biological functions, for example mitogenic, pro- and anti-apoptotic. MMPs and TIMPs are secreted both by tumor-associated stromal cells and by cancer cells and are important components of the tumor microenvironment. Overexpression of MMPs and TIMPs was reported for almost all tumor types and associated with a bad survival prognosis.

In the present study we focused our investigations on the effect of the microtubule stabilizing agent patupilone on the MMP function in tumor cells after treatment with ionizing radiation. The results presented herein further confirmed the radiosensitizing capacity of patupilone to enhance IR-induced cytotoxicity in a wide range of human cancer cells (HCT116 colon carcinoma cells, HT1080 fibrosarcoma cells, U251 glioma cell and medulloblastoma

cell lines DAOY, D425, D341). It is also interesting to note that patupilone sensitized to ionizing radiation already in a very low dose range. At these doses patupilone was not cytotoxic itself and did not alter microtubule cytoskeletal structures (0.2 nM patupilone in HT1080 cells). The combined treatment modality with patupilone and IR is more potent *in vivo* than in cellular model systems. Most probably, this is due to the additional treatment effects on the level of the tumor microenvironment, which may be mediated by tumor cells. Herein, we investigated an effect of the indicated treatment regimens on one of the important components of the tumor microenvironment, namely MMPs. We identified that the activity of secreted MMP was upregulated after treatment with IR and that pre-treatment with patupilone could diminish this increase. MMP activity is required for tumor cell invasion as well as for other cellular processes. In this study tumor cell invasiveness was indeed increased after IR and, in line with the events on the level of MMP activity, patupilone could counteract this effect of IR.

In an attempt to uncover mechanisms that contribute to the counteracting effect of patupilone on IR-induced MMP activity, we dissected the MMP regulation cascade on vaious levels. Patupilone did not influence IR-induced events on the level of cell cycle distribution and gene transcription. Namely, patupilone did not interfere with the accumulation of cells in G2/M phase after IR and did not alter IR-induced transcription of MMPs and TIMPs. Both MMP and TIMP protein levels were elevated in the cell lysate and conditioned cell culture media (CM) after treatment with 10 Gy of IR. Patupilone did not influence intracellular and extracellular protein levels of MMPs. However, the amount of extracellular TIMP-1 and TIMP-2 proteins after combined treatment with patupilone and IR was significantly reduced when compared to treatment with IR alone. Furthermore, the counteracting effect of patupilone on IR-induced MMP activity was not observed in TIMP-1 and TIMP-2 depleted cells. After TIMP depletion the IR-increased MMP activity did not exceed the MMP activity level after combined treatment in non-depleted cells, indicating that the proMMP activation processes after IR requires TIMPs. These findings strongly suggest that patupilone counteracted IR-induced MMP activity due to the interference with the MMP activation processes by the depletion the secreted TIMP-1 and TIMP-2 proteins.

To our knowledge, we demonstrate for the first time that patupilone can counteract IR-induced MMP-related processes. It is particularly interesting is that the counteracting effect of patupilone was observed in human glioma cells. Glioblastoma (or grade IV glioma) is one of the most treatment resistant tumors in adults. The primary reasons associated with the poor prognosis are: extensive infiltration of surrounding brain tissue by tumor cells and the blood-brain barrier as an obstacle for the adequate delivery of chemotherapeutics. The ability of patupilone to cross the blood-brain barrier makes patupilone a very potent and promising chemotherapeutical agent for the treatment of brain malignancies. The capacity of patupilone to inhibit IR-induced glioma cell invasion, as shown in the present study, increases the value

of the compound as a candidate for a combination with radiation therapy for glioblastoma treatment.

MMPs play an important role in cancer progression by remodelling the extracellular matrix and regulating multiple biologically active molecules. Overexpression of MMPs and TIMPs in multiple tumor types positively correlates with the aggressiveness of the disease. There is a strong indication that radiation therapy can promote MMP expression and tumor invasiveness. Thus, therapeutic agents that cleverly target members of MMP and TIMP families in selective and disease/function-specific way can represent an interesting class of agents for a combination with ionizing radiation.

This study demonstrates that the MTSA patupilone is a compound of great interest. It is a potent inhibitor of tumor cell proliferation under hypoxic and normoxic conditions, a very promising anti-metastatic and anti-angiogenic agent, a potent radiosensitizer *in vivo* and *in vitro* (as described elsewhere). In addition, the present study demonstrated that patupilone inhibits IR-induced activity of secreted MMPs and IR-induced cell invasion. Since IR-induced MMP activity may be relevant to cancer progression, the combined treatment modality of IR and patupilone might, indeed, be of great clinical benefit for cancer therapy.

Zusammenfassung

Die Kombinationstherapie von ionisierender Strahlung mit dem neuen, klinisch relevanten Microtubuli-stabilisierenden Agens (MSTA) Patupilone ist eine vielversprechende Behandlungsmethode gegen Krebs, die in unserem Labor auf präklinischer Ebene untersucht wird. Patupilone (Epothilone B), welches aus Bakterien gewonnen wird, bindet an der gleichen Stelle an β-tubulin wie Taxane. Im Gegensatz zu Taxanen ist Patupilone aber auch in multidrug resistenten Tumoren wirksam und wegen seiner einfachen chemischen Struktur existieren Derivate mit verbesserten pharmakokinetischen Eigenschaften. Die Kombination von Patupilone und ionisierender Strahlung zeigt eine stark supra-additive Hemmung des Tumorzellwachstums in Tumor Xenografts in Mäusen. Interessanterweise ist dieser ergänzende Effekt viel geringer in vitro, was darauf schliessen lässt, dass die gemeinsame Behandlung von ionisierender Strahlung mit Patupilone einen zusätzlichen Effekt auf die Tumorumgebung ausübt. Zu Beginn wurde davon ausgegangen, dass Patupilone einen direkten Effekt auf neovaskuläre Endothelialzellen hat. Nachdem aber festgestellt wurde, dass dieser additive Effekt in vivo nur in Patupilone sensitiven Tumorzellen zu beobachten ist, wird angenommen, dass Patupilone einen indirekten Effekt auf die Tumorumgebung ausübt, der über Tumorzellen vermittelt wird, z.B. Sekretion von biologisch aktiven Faktoren.

Matrix Metalloproteinasen (MMPs) sind Zink-abhängige Endopeptidasen mit einem breiten Spektrum an Substraten, zu denen Komponenten der extrazellulären Matrix und Wachstumsfaktoren gehören (Transforming growth factor β (TGF-β). MMPs spielen eine wichtige Rolle für Zellüberleben, Apoptose, Angiogenese, Matrix Modulierung und in der Metastasenbildung. Die MMP-Aktivität ist durch einen komplexen Aktivierungsmechanismus (MMPs werden als Zymogene sekretiert) und durch natürliche Inhibitoren reguliert. Die Tissue inhibitors of matrix metalloproteases (TIMPs) sind eine Familie von vier kleinen Proteinen (20-30kDa). TIMPs sind die wichtigsten MMP-Regulatoren die man bis anhin kennt. Alle 4 TIMPs inhibieren die enzymatische Aktivität von MMPs. Zudem konnte gezeigt werden, dass TIMP-2 in der MMP-2-Zymogen-Aktivierung involviert ist. Zusätzlich zur Regulierung von MMP-Aktivität haben TIMPs auch MMP-unabhängige Funktionen, z.B. mitogene, pro- und anti-apoptotische Funktionen. MMPs werden von Tumor- und Tumor-assoziierten Stromazellen sekretiert und stellen einen wichtigen Bestandteil der Tumorumgebung dar. Überexprimierung von MMPs und TIMPs konnte in fast allen Tumorentitäten festgestellt werden und geht mit einer schlechten Überlebensprognose einher.

Im Fokus der vorliegenden Arbeit stand daher die Untersuchung der Wirkung des Mikrotubuli stabilisierenden Agens Patupilone auf die MMP-Aktivität in bestrahlten Zellen. Die Resultate der Arbeit zeigen, dass Patupilone zu einer erhöhten Zytotoxizität in be-

strahlten Zellen führt. Dies wurde schon früher berichtet und konnte hier in verschiedenen Zelllinien bestätigt werden (HCT116 Coloncarcinom Zellen, HT1080 Fibrosarcom Zellen, U251 Glioma Zellen und in den Medulloblastom Zelllinien DAOY, D425, D341). Von spezieller Bedeutung ist die Tatsache, dass Patupilone schon in nicht-zytotoxischen Dosen, welche die Mikrotubulistruktur nicht verändern, diesen Effekt zeigt (0.2nM in HT1080). Wie schon erwähnt, ist die gemeinsame Behandlung mit Patupilone und ionisierender Strahlung wirksamer in vivo als in vitro, weshalb davon ausgegangen wird, dass ein zusätzlicher, indirekter Effekt von Tumorzellen auf die Tumorumgebung stattfindet. In dieser Arbeit wurde daher der Effekt von Patupilone in Kombination mit ionisierender Strahlung auf MMPs, eine wichtige Komponente der Tumorumgebung, untersucht. Wir konnten zeigen, dass Bestrahlung zu erhöhter MMP-Aktivität führt und dass die Behandlung mit Patupilone diesen Anstieg der MMP-Aktivität hemmt. MMP-Aktivität wird auch für die Zellinvasion benötigt und daher wird auch die strahleninduzierte Invasion durch die Behandlung mit Patupilone inhibiert. Um mehr über den Mechanismus zu erfahren, durch den Patupilone MMP-Aktivität und Invasion inhibieren kann, wurde die MMP-Regulierung auf verschiedenen Ebenen untersucht.

Es konnte gezeigt werden, dass Patupilone die strahleninduzierten Prozesse nicht durch Veränderungen in der Zellzyklusverteilung oder der Gentranskription beeinflusst (Patupilone hatte keinen Einfluss auf den strahleninduzierten G2/M Phase Arrest und veränderte auch die Transkription von TIMPs und MMPs nicht). Sowohl MMP als auch TIMP Proteinmengen waren erhöht in Zelllysaten und in konditioniertem Medium nach Bestrahlung mit 10 Gy. Patupilone hatte keinen Einfluss auf die intra- und extrazellulären MMP Proteinmengen. Interessanterweise aber waren die extrazellulären Proteinmengen von TIMP-1 und TIMP-2 signifikant niedriger in Zellen die kombiniert mit Strahlung und Patupilone behandelt wurden, als in Zellen die nur bestrahlt wurden. Des Weiteren konnte gezeigt werden, dass der inhibitorische Effekt von Patupilone auf strahleninduzierte MMP-Aktivität in TIMP herunterregulierten Zellen nicht mehr beobachtet werden konnte. In Zellen, in welchen TIMP Proteine herunterreguliert wurden, war die Aktivierung von MMP nach Bestrahlung in gleichem Ausmass inhibiert wie in Zellen, in denen die TIMP Proteine nicht herunterreguliert wurden. Diese Daten weisen stark darauf hin, dass strahleninduzierte MMP Aktivierung TIMP abhängig ist und dass Patupilone diese strahleninduzierte MMP Aktivierung durch die Reduktion von extrazellulärem TIMP-1 und TIMP-2 inhibieren kann.

Nach unserem Wissen sind wir die Ersten die zeigen können, dass Patupilone strahleninduzierte Aktivierung von MMPs hemmen kann. Von spezieller Bedeutung ist, dass dieser Effekt von Patupilone in menschlichen Gliomazellen gezeigt werden konnte. Glioblastome (oder Grad 4 Glioma) gehören zu den resistentesten Tumoren in Erwachsenen. Der Grund für die hohe Resistenz ist die starke Infiltrierung von Hirngewebe durch die Tumorzellen und die Blut-Hirn-Schranke, welche sich als Barriere für viele Chemotherapeutika erwiesen hat. Patupilone aber kann die Blut-Hirn-Schranke passieren. Die Fähigkeit von Patupilone,

die Invasivität von Glioblastomen zu hemmen, erhöht das klinische Potential dieses Agens zusätzlich und macht es zu einem vielversprechenden Therapeutikum in der Krebsbehandlung in Kombination mit Bestrahlung.

MMPs spielen eine wichtige Rolle im Fortschreiten von Krebs durch Veränderung der extrazellulären Matrix und multipler bioaktiver Moleküle. Überexprimierung von MMPs und TIMPs in verschiedenen Tumorentitäten geht einher mit der Aggressivität der Erkrankung. Viele Berichte deuten darauf hin, dass die Strahlentherapie zu einer erhöhten MMP-Aktivität führen und die Invasivität von Tumoren fördern kann. Daher könnten Therapeutika, welche spezifisch auf Elemente der MMP und TIMP Klasse wirken, vielversprechend in der Krebstherapie in Kombination mit Bestrahlung sein.

Diese Arbeit konnte aufzeigen, dass das Mikrotubulistabilisierende Agens Patupilone von grossem Interesse für die Krebsbehandlung ist. Es ist ein wirksamer Inhibitor von Tumorzellmigration und Tumorzellwachstum unter hypoxischen und normoxischen Bedingungen. Es konnte gezeigt werden, dass Patupilone einen radiosensitivierenden Effekt in vitro und noch stärker in vivo hat. Zusätzlich konnte gezeigt werden, dass Patupilone stahleninduzierte MMP-Aktivität und Invasion inhibiert. Da strahleninduzierte MMP-Aktivität möglicherweise eine Rolle in der Krebsfortschreitung spielt, könnte die Kombination von Patupilone mit Bestrahlung tatsächlich zu einem grossen Fortschritt in der Krebsbekämpfung führen.

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List of Abbreviations

AB	antibody
AP-1	activator protein-1
Aph	aphidicoline
APMA	4-aminophenylmercuric acetate
CM	conditioned cell culture media
DAPI	4’,6-diamidino-2-phenylindole
DDR	DNA damage repair
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB	double-strand breaks
ECM	extracellular matrix
EGFP	enhanced green fluorescent protein
EMT	epithelial-to-mesenchymal transition
FACS	fluorescence activated cell sorting
FASL	FAS death receptor ligand
FCS	fetal calf serum
FDA	the Food and Drug Administration
FGR	fibroblast growth factors
FRET	fluorescence resonance energy transfer
GDP	guanosine diphosphate
GF	growth factor
GTP	guanosine triphosphate
Gy	Gray
HIF	hypoxia-induced transcription factor
HPV	human papiloma virus
HR	homologous recombination
HU	hydroxyurea
IC	inhibitory concentration
IGF	insulin growth factor
IL	interleukin
IR	ionizing radiation
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
mRNA	messenger ribonucleic acid
MT	microtubule
MT-MMP	membrane-type matrix metalloproteinase
MTOC	microtubule organizing center

MTSA	microtubule stabilizing agent
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
MVD	microvessel density
nAB	neutralizing antibody
NEAA	non-essential amino acides
NF-kB	nuclear factor kB
NHEJ	non-homologous end-joining
NNGH	N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid
NSCLC	non-small cell lung cancer
PBS	phosphate buffered saline
PE	plating efficiency
PI3K	phosphoinositide 3-kinases
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate (phorbol ester)
pO_2	partial pressure of oxygen
qRT-PCR	quantitative real time polymerase chain reaction
RCE	retinoblastoma control element
RECK	reversion-inducing-cysteine-rich protein with Kazal motifs
RNA	ribonucleic acid
RT	radiation therapy
SDS	sodium dodecyl sulfate
SE	standard error
SF	surviving fraction
siRNA	small interfering ribonucleic acid
TAM	tumor-associated macrophages
TBP	TATA box-binding protein
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinases
TME	tumor microenvironment
VEGF	vascular endothelial growth factor
WHO	the World Health Organization

1 Introduction

1.1 Cancer and cancer treatment

1.1.1 Cancer: epidemiology and management

Cancer is the common name for a group of more than 100 diseases generally characterised by uncontrolled cell proliferation and spread. Cells in almost every tissue of the body can give rise to cancer, and malignancies can be classified by site of origin: carcinomas – cancers of epithelial origin, sarcomas originate from mesenchymal tissue, leukemias – from hematopoietic cell lineage etc. When the growing cancer cell mass disrupts essential tissues and organs, the disease becomes lethal. According to the World Health Organization cancer causes 20 % of deaths in the European region. With more than 3 million new cases and 1.7 million deaths each year, cancer remains the most important cause of death and morbidity in Europe after cardiovascular diseases. The most frequent cancers among men are lung, stomach, liver, colorectal, oesophagus and prostate; among women – breast, lung, stomach, colorectal and cervical (Fig 1.1, 1.2). A study on cancer epidemiology performed in 2008 showed that trends in incidence are actually generally favourable in the more prosperous countries from Northern and Western Europe, except for obesity related cancers and, for women, tobacco-related cancers [1]. However, worldwide prognosis is not that favourable and according to the WHO estimation (done in 2004) deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. The unfavorable trends are mainly attributed to low-income countries, which have no adequate programs for cancer prevention and

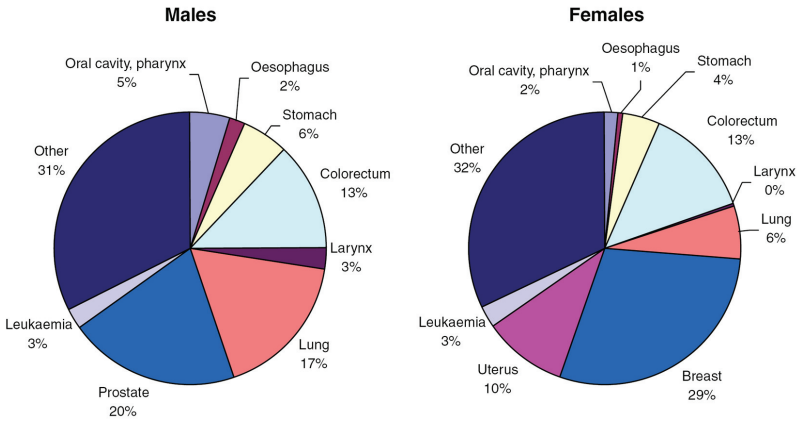

Figure 1.1. Distribution of the cancer cases by gender in Europe in 2006 [1]

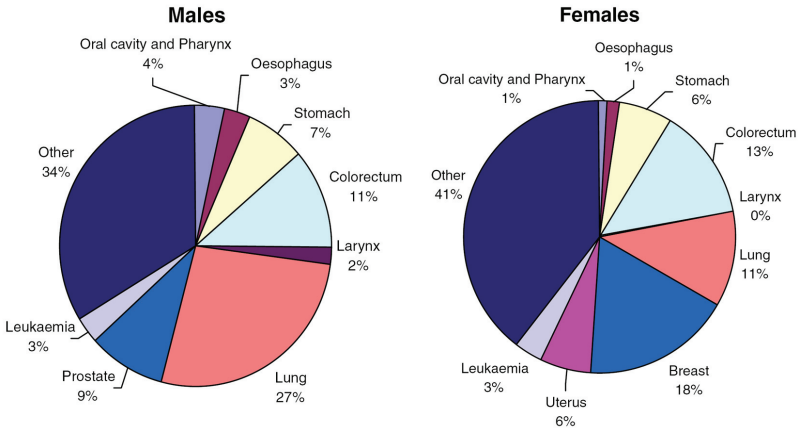

Figure 1.2. Distribution of cancer death by gender in Europe in 2006 [1]

treatment.

Cancer prevention includes healthy lifestyle and early diagnosis as some of the most common tumor types, including breast, colorectal and cervical cancer, can be cured if detected early. The three gold standard treatment strategies for cancer include surgery, radiotherapy and chemotherapy. *Surgery* is the oldest form of cancer treatment. About 60 % of all cancer patients will undergo surgery, which simply involves the removal of a malignant tumor. It works best on localized cancers that haven't yet spread to other parts of the body, and is often followed by radiation therapy or chemotherapy to ensure that all malignant cells have been removed.

The other two treatment modalities are capitalizing on the fact that tumor cells are different from normal cells in terms of their proliferation rate and are therefore are much more sensitive to genotoxic stress. *Radiation therapy* is given to over 50 % of cancer patients. The modern, state of the art irradiation techniques allow to deliver very high radiation doses specifically to the tumor, while substantially sparing the surrounding healthy tissue. *Chemotherapy* is the general term for any treatment involving the use of chemical agents to prevent the growth of cancer cells. It allows to treat systemic oncological diseases, such as lymphomas, and the elimination of cancer cells that have already been involved in the metastatic process. More than half of all people diagnosed with cancer receive chemotherapy. Very often different treatment strategies are combined to achive the best treatment outcome.

1.1.2 Tumor biology

Over the previous decades, a complex body of knowledge was generated, which brought a substantial understanding of cancer biology. At the very end of the 20th century, D. Hanahan and R. Weinberg [2] formulated a set of rules that govern the malignant transformation. They highlighted six hallmark capabilities (Fig. 1.3) that a normal cell must acquire to become a malignant cell:

(1) *"Self-sufficiency in growth signals"*: In contrast to normal cells, cancer cells do not

depend on external mitogenic growth factors (GF) for proliferation. This can be achieved, for example, by overexpression of GF receptors (e.g. the epidermal GF receptor Erb) or by constitutive activation of members of growth signaling pathways (e.g. mutations in ras gene) (2) *"Insensitivity to growth-inhibitory signals"*: Cells in a normal tissue are maintained quiescent either by antigrowth signals that force them to exit cell cycle or by acquisition of a differentiated state. Cancer cells can become desensitized to these signals by downregulation of responsible genes, such as pRb protein – negative regulator of cell cycle progression, or overexpression of proteins to avoid differentiation, for example c-Myc. (3) *"Evasion of programmed cell death (apoptosis)"*: Apoptosis can be triggered in a cell as a response to abnormalities (for example a signaling imbalance), and therefore it is important for cancer cells to learn to evade it. This can be achieved through the upregulation of anti-apoptotic proteins (Bcl-2) or downregulation of the function of pro-apoptotic regulators (mutations in p53). (4) *"Limitless replicative potential"*: Normal cells have the capacity for 60-70 doublings due to telomere shortening. As many cells in the tumor are dying due to unfavorable mutations and harsh environmental conditions, the surviving cancer cells must acquire the ability to multiply infinitely to increase tumor mass. Cancer cells acquire limitless replicative potential by over-expressing the telomerase enzyme. (5) *"Sustained angiogenesis"*: To be able to progress to a larger size, tumor must provide itself with a sufficient nutrient and oxygen supply. In order to do so, cancer cells must develop an angiogenic ability that seems to be acquired by changing the balance of pro- and anti-angiogenic factors – the so-called "angiogenic switch". The tumor environment seems to play an important role in this process, as will be discussed in more details in chapter 1.1.3. (6) *"Tissue invasion and metastasis"* is one of the latest features that are acquired by cancer cells. The requirements for successful cell invasion include: first, changes in physical interaction with the environment; second, acquisition of a sufficient level of motility; and, last, the ability to degrade the extracellular matrix (ECM). Cancer cells alter the regulation of

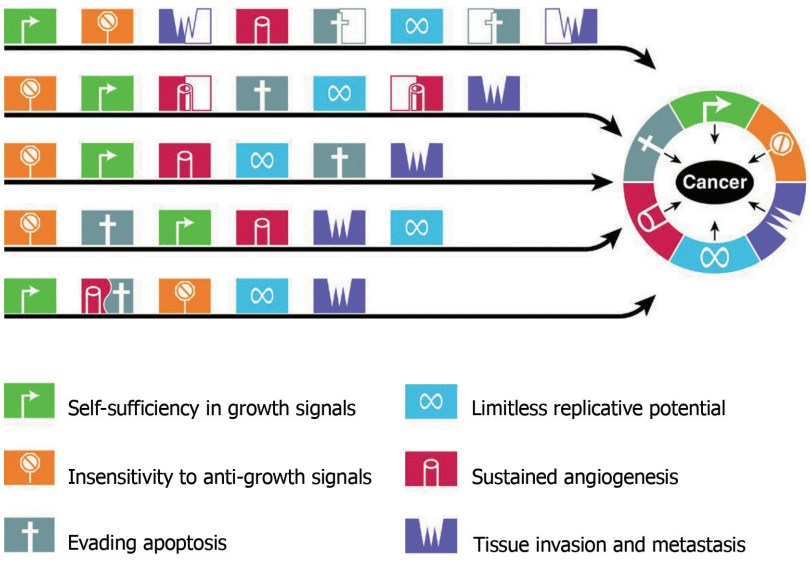

Figure 1.3. Pathways of tumorigenesis. The six hallmark capabilities of cancer (self-sufficiency in growth factors, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis) are acquired in variable order across the spectrum of cancer types and subtypes. Adapted from [2].

cell-cell and cell-matrix adhesions by means such as deactivating the E-cadherin and changing the integrin isoform composition. Tumor cells also over-express extracellular proteases (matrix metalloproteinases) for extensive ECM degradation [2].

These hallmark capabilities should be acquired through genetic mutations. It is considered that “mutator mutations” must occur that lead to so-called genetic instability – an enhanced rate of changes in nucleotide sequence or of chromosomal rearrangements. The genetic instability, in turn, provokes further mutations, which can potentially lead to pro-malignant changes and “mutator phenotype”. The “mutator mutations” are most likely to be in genes that are important for DNA damage repair and cell cycle checkpoints. They can be inherited or occur sporadically over a life span as a result of exposure to environmental factors and aging [3].

1.1.3 Tumor microenvironment

The tumor is a complex organ-like structure with cancer cells and stromal cells being in close interaction. Tumor associated stromal cells are recruited from surrounding normal tissue or from circulation. Classically recognized components of the tumor stroma include connective tissue, blood vessels, inflammatory cells and fibroblasts [4]. The tumor stroma provides growth factors and cytokines, thereby influencing the proliferation and invasion of tumor cells, angiogenesis and metastasis. Physical characteristics of the tumor microenvironment (TME) also play an important role in cancer progression. One of them is tumor hypoxia – a condition that results from an imbalance of oxygen delivery and oxygen consumption in the tumor and is characterized by the low oxygen content in the tumor. Hypoxia induces defined pattern of specific gene transcription, which leads to an adaptation ofthe tumor to low oxygen condition. The hypoxia-induced transcription factor-1α (HIF-1α) upregulates the expression of pro-angiogenic proteins, such as the vascular endothelial growth factor (VEGF). Moreover, the HIF-1α signalling pathway is important for tumor cell dissemination [5-6] and the survival of tumor cells at the metastatic site [7].

Secreted proteins are another essential component of the TME. One example is the aforementioned VEGF, which is secreted by tumor cells and induces the proliferation of endothelial cells during angiogenesis. Another example is secreted proteases, such as MMPs (matrix metalloproteinases), which participate in multiple tumor-related processes (including metastatic invasion and angiogenesis) by controlling bioavailability of the regulatory factors. Specific tumor types are associated with a unique tumor stroma. For example 50 % of an invasive breast tumor’s mass may consist of tumor-associated macrophages (TAMs); similarly, fibroblasts are the predominant component found in the stroma of colon cancer [8]. Multiple studies have shown that unique stromal elements (such as cancer-associated fibroblasts and TAMs (rev in [4]), as well as tumor hypoxic status [9] correlate with poor disease prognosis. The tumor-associated stromal cells can contribute to the treatment response by sup-

porting cancer cell survival. However, as they are more genetically stable than tumor cells, they themselves should be less prone to develop drug resistance. Therefore targeting stromal cells (e.g. angiogenic vessels) or their products (for example, VEGF) is a good strategy for cancer therapy and for overcoming the problem of treatment resistance [10-11].

1.1.4 Cell migration and metastasis

Cancer cells possess a broad spectrum of migration and invasion mechanisms. These include both individual and collective cell migration strategies. Generally speaking, invasion requires migration, adhesion and proteolysis of extracellular matrix components. First, the moving cell becomes polarized and elongates. The cell protrudes its leading edge, which attaches to the ECM substrate. Subsequently, regions of the leading edge or the entire cell body contract, thereby generating traction force that leads to the gradual forward gliding of the cell body and its trailing edge. The migration of cells is a cyclical process that requires interplay between the microtubule and actin cytoskeletal systems. The microtubule growth occurs at the leading edge and microtubule shortening occurs at the cell body and rear. Microtubule growth and shortening may activate Rac1 and RhoA signalling respectively, to control actin dynamics. Rac1 activity at the leading edge promotes actin assembly at the leading edge. RhoA activity in the cell body drives actomyosin contractility and the formation of actin bundles and focal adhesions, increasing contractility in the direction of migration, possibly causing further microtubule breakage in the cell body. Thus, the dynamic interactions between microtubules and actin may provide a positive feedback loop to drive cell migration [12].

The turnover of adhesions is critical for affective migration. Integrins come into contact with ECM ligands and cluster in the cell membrane. Clustered integrins recruit adaptor and signalling proteins (like α-actinin, the focal adhesion kinase (FAK), vinculin, paxillin), forming the adhesion focal complexes. The adhesions provide the required traction for the cell to pull forward. At the same time, disassembly of the adhesion complexes at the rear of the cell is necessary to enable forward progress. As a result of ECM sensing, surface proteases become

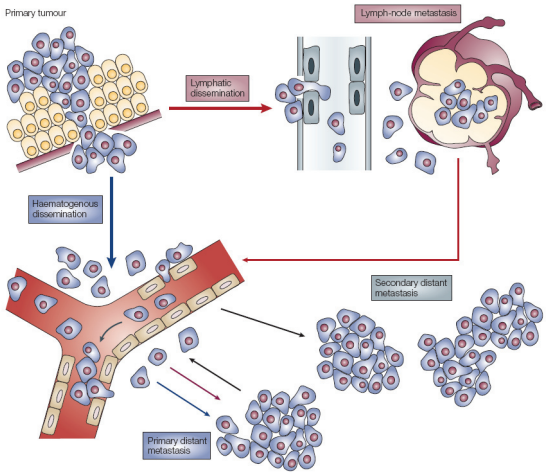

Figure 1.4. Metastatic process. Cancer cells can disseminate from the primary site via lymphatic routes (red arrows) and by haematogenous routes (blue arrows). Secondary haematogenous dissemination also occurs from overt metastases to other distant sites (black arrows) [16].

concentrated near substrate binding sites. In close proximity to the cell surface, proteases cleave ECM components (such as collagen, fibronectin and laminins) as well as pro-MMPs to create active soluble MMPs, such as MMP-2. MT1-MMP, MMP-1 and other collagenases cleave native collagens, along with other ECM macromolecules, into smaller fragments. These in turn, are accessible to subsequent degradation by gelatinases (MMP-2 and MMP-9) or serine proteases [13].

The process of tumor cell dissemination and metastasis is understood as the migration of individual cells, which detach from the primary tumor, enter lymphatic vessels or the blood-stream and seed in distant organs (Fig. 1.4). Mechanical factors influence the initial fate of disseminating cancer cells. Blood flow determines to which organ the cancer cell will travel first. As the relatively large size of tumor cells does not allow them to pass through capillaries, the disseminating cells get arrested in the first capillary bed they encounter. However, the ability of the cancer cell to proliferate at the metastatic site is determined by the molecular interactions with the organ environment. The “seed (cancer cell) and soil (factors in the organ environment)” theory claims that as a result of these interactions, tumors have an organ-specific pattern of metastasis. For example, breast cancer frequently metastasizes to bone, brain and lungs; prostate cancer preferentially spreads to bone [14]. Due to the complexity of the process and multiple challenges for the tumor cell (such as survival in a blood stream and growth in a foreign environment) metastatic process is highly inefficient. Nevertheless, it is the main cause of cancer-related morbidity, as cell invasion has proved to be difficult to block safely and effectively with pharmacological compounds [13, 15].

1.2 Radiation therapy

1.2.1 Mechanisms of cytotoxicity

Conventional radiation therapy is usually applied with a beam composed of x-rays (photons). When radiation initially interacts with biologic matter, direct or indirect ionizations take place. The mechanism of ionization by x-rays is deemed “indirect” when the effects are caused by secondary particles. Ionizing radiation gives rise to fast (high energy), charged secondary particles (electrons), which can then directly or indirectly cause ionizations in the critical target molecules (e.g. DNA). The predominant interaction usually takes place with the most abundant cellular medium, water. The series of reactions that will follow are known as radiolysis of water and result in production of free radicals. In the presence of oxygen, the free radicals will produce RO_2, organic peroxides (reactive oxygen species – ROS), which are highly reactive. In a cell, IR may damage almost any components, including proteins and lipids in the cell membrane [17-18]. DNA damage, however, is the most relevant for cell killing [19].

Damage to DNA can be classified into a number of general classes that include dimers,

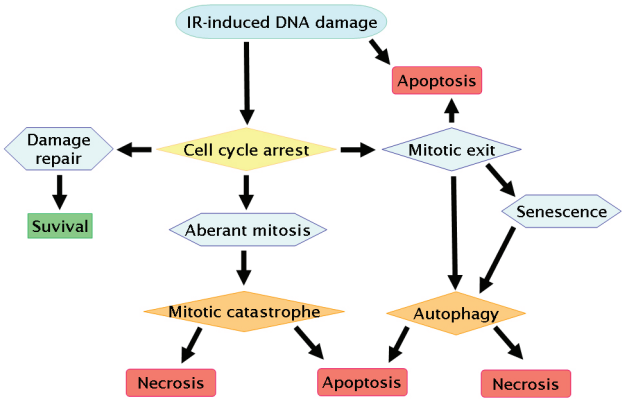

Figure 1.5. Cell fate after IR-induced DNA damage.

adducts, oxidative base damage, intra- and inter-strand crosslinks, and single and double-strand breaks (DSB). To be able to repair the DNA damage a cell has mechanisms for DNA damage sensing that activate signal transduction pathways, which in turn orchestrate multiple cellular functions for successful damage repair. DSBs are the most complex lesions and therefore they are the greatest threat for cell survival as the lack of an intact template DNA strand makes repair of the DSB difficult and error-prone. The two well-described DSB repair strategies are termed homologous recombination (HR) and non-homologous end-joining (NHEJ). The fundamental difference between the two is the dependence on DNA homology in the former. Therefore HR plays a more prominent role during late S and G2 phases of the cell cycle, when the sister chromatid DNA strands are available. NHEJ uses little to no sequence homology for repair and therefore it is more important during G1 and early S phase [20].

Radiation leads to cell death by way of different mechanisms (Fig. 1.5). Even low doses of irradiation, as a stress factor, can induce fast apoptosis in interphase in some cells. Apoptosis is an active process characterized by programmed cell death in which a defined cascade of events takes place. Various factors may modulate the apoptotic response to DNA damage, including the p53 status of the cell and levels and activity of the pro- and anti-apoptotic proteins. The ability to evade apoptosis is one of the features exhibited by cancer, however, there seems to be no direct correlation of apoptosis and radiosensitivity. A basic principle in the radiobiology of tumors is that an essential difference exists between cell death and the loss of reproductive integrity of tumor cells, so-called clonogenicity, and for the outcome of curative RT the latter matters most [21].

If cells do not undergo apoptotic death in interphase, they may become arrested in G1 and/or G2 phases of the cell cycle and then, as soon as the repair processes are accomplished, continue to proliferate. However, if the DNA damage was not sufficiently repaired during the arrest, the fate of the cell develops according to different scenarios that lead to either cell death or to loss of clonogenicity. The possible mechanisms include: (i) mitotic catastrophe – a result of aberrant mitosis, characterized by formation of nuclear envelopes around individual clusters of missaggregated chromosomes; (ii) senescence – a state when cells loose their abil-

ity to proliferate but keep on metabolizing or (iii) autophagy – another type of programmed cell death which is characterised by the appearance of autophagic vacuoles in the cytoplasm. The cells death via mitotic catastrophe can have phenotypic sings of apoptosis or necrosis (a passive cell death process, characterised by disintegration of nuclear and cellular envelope) [21-24].

Radiotherapy (RT) is one of the most prevalent cancer treatments. The goal of radiotherapy is to increase the total radiation dose to tumor tissue, while simultaneously limiting the toxicity in surrounding normal tissues to a tolerable degree. This goal can best be achieved by high accuracy conformal techniques and a sensitive fractionation schedule, where the total dose is applied in multiple (up to 35) small daily fractions. Fractionation helps to reduce dose limiting toxicities, by employing the major differences between cancer and normal cells: most cancer cells are DNA damage repair (DDR)-impaired and proliferate more rapidly than most normal cells. It has been speculated that DDR inhibition might enhance the effectiveness of radiotherapy and DNA-damaging chemotherapies. Indeed, various DDR-inhibitory drugs are in preclinical and clinical development to test this premise [25].

1.2.2 Unfavourable effects of radiation therapy: focus on metastasis

In spite of the persistent effort to enhance the precision of modern radiation therapy, normal tissue toxicity still has to be taken into consideration. Nowadays, acute side effects of RT are generally clinically manageable but late side effects (such as secondary tumors at the irradiated site) remain an important issue especially for treatment of childhood cancers [26]. Another aspect, which is regularly reported in literature, is the potential pro-metastatic effect of IR. However, there are no comprehensive studies of this problem and it is difficult to draw a firm conclusion from the existing reports because of the heterogeneity of patient cohorts (age, race and gender, tumor sites, staging), treatments applied (dose and fractionation of RT, target of RT, combination with surgery and chemotherapy), response to treatment (local failure or local control), diagnosis of metastasis (including autopsy or not) and methods of statistical analysis. The overall impression however remains that under certain circumstances RT favors metastasis. The experimental evidence occur that ionizing radiation can upregulate metastasis-related processes [27].

IR was shown to induce matrix metalloproteinases (MMPs, see Chapter 1.4) and cell invasion, not only in *in vitro* assays [28-31], but also in *in vivo* mouse models. The use of radiation for treating primary murine lung (LLC-LM) tumors resulted in the growth of previously dormant lung metastases [32]. Fractionated irradiation of prostate cancer xenografts in mice led to local tumor control but increased disseminated metastases, whereas non-irradiated local xenografts failed to disseminate. In this study a correlation was found between the appearance of irradiation-induced metastases and enhanced activity of MMP-9 in the urine of irradiated mice [33]. Though the activation of MMPs by IR is a well-established

fact, pro-invasive action of IR is still a topic of discussion. IR has been shown to decrease cell invasion *in vitro* [34-35] and in pre-clinical studies, for example IR decreased the occurrence of lung metastasis in mice bearing mammary carcinoma. The role of radiation treatment in metastatic processes is a matter of great debate and seems to be strongly dependent on the tumor type and the experimental system that was used in the study. However, the pro-metastatic action of RT may explain why the better local control achieved by this treatment modality fails to be translated into longer survival, free of distant metastasis, and that the better radiotherapeutic strategies must aim at a better metastasis control [27].

1.3 Microtubules as a target for cancer treatment

1.3.1 Tubulins

α- and β-tubulins are the two families of the tubulin superfamily that were discovered first and have been since studied in most detail. They form heterodimers that build microtubules (MT) (Fig. 1.6). Seven known isotypes of β-tubulin are mainly tissue specific: βI- and βIVb-tubulins are constitutively expressed in main cell types; βII is a major isotype in neurons; βIII is also found in neurons and increased in several tumor types being associated with aggressive and invasive disease; βIVa is brain specific; βV is specific for uterus and endometrium and was found in adenocarcinomas; and βVI-tubulin is located in hepatopoesis-specific cells. The tubulin isotypes within one tubulin family share a high degree of homology and differ in the C-terminal region of 20-27 amino acids. Further members of the tubulin family include the γ-tubulin (a protein, which plays a role in microtubule nucleation) and the four recently

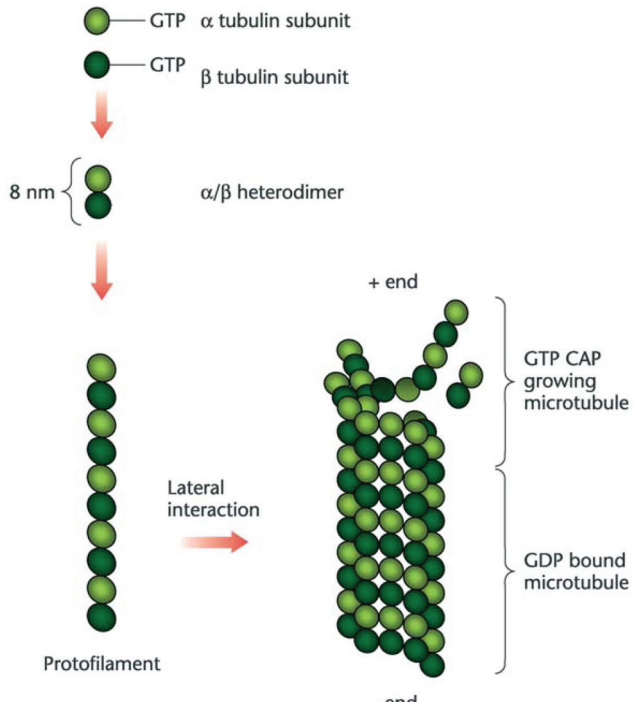

Figure 1.6. Structure of microtubules. α- and β-tubulin heterodimers are associated end-to-end into a protofilament. Protofilaments side-by-side packing forms the wall of the microtubule [43].

discovered tubulins – delta (Δ)-, epsilon (ε)-, zeta (ζ)- and eta (η)-tubulin. The functions and localization of the four "new" tubulins are associated with the eukaryotic centrosomes and other MT organizing centers (MTOCs) [36]. α- and β-tubulins are subject for a number of translational modifications including acetylation, tyrosination, phosphorylation, polyglutamilation and polyglycylation. Most α-tubulins are known to be acetylated in stabilized MTs after assembly. Stable microtubules also appear to be more detyrosinated. A substantial tubulin and microtubule diversity is created due to tubulin post-translational modifications together with multiple tubulin isotypes, but the biological meaning of this diversity is not yet well understood [37].

There are known β-tubulin mutations that lead to insensitivity of cells to MT-interfering cytotoxic drugs. These mutations can be obtained as a result of directed cell selection. For example, the Gln to Glu mutation at residue 292 of βI-tubulin in A549 human lung tumor cells led to a phenotype 100-fold more resistant to epothilone B [38], and the Asp to Glu mutation at residue 26 resulted in a phenotype 20-fold more resistant to paclitaxel [39]. However, spontaneous mutations of this kind are typically rare events and therefore not clinically relevant [40]. What really causes insensitivity of tumor cells to therapy with MT-interfering drugs is a change in the isotype composition of tubulins, which forms microtubules in cancer cells [41]. Overexpression of βIII-tubulin is associated with resistance of ovarian, breast, NCLC and other cancer types to both, the MT-stabilizing compounds from the taxan group and the MT-destabilizers vinca alkaloids. Overexpression of "harmless" tubulin types, like βI and βII, can also lead to drug-resistance (reviewed in [42]).

1.3.2 Microtubules and their cellular functions

Microtubules are polymeric molecules built of α- and β-tubulin heterodimers. The heterodimers are associated end-to-end into a protofilament. Their side-by-side packing forms the wall of the microtubule (usually 13 filaments, form a cylindrical microtubule with an outer diameter of 25 nm) (Fig. 1.6). The microtubules display structural polarity in which the addition of subunits occurs preferentially at one end designated the (+) end. The other end is called the (-) end and is usually oriented towards MTOC [44-45]. Microtubules are highly dynamic and can switch stochastically between phases of growth and shortening. This non-equilibrium behaviour, known as dynamic instability, is based on the binding and hydrolysis of GTP by tubulin subunits. Each tubulin monomer binds one molecule of GTP. The binding of GTP to α-tubulin at the N-site is non-exchangeable, whereas the binding to β-tubulin at the E-site is exchangeable. Only tubulin dimers with GTP in their E-site can polymerize, but after polymerization this nucleotide is hydrolyzed and becomes non-exchangeable. The most favoured hypothesis to explain dynamic instability is the GTP cap model. In this model, the body of the microtubule is made of GDP-tubulin subunits and is therefore unstable. The microtubule structure is only stabilized by a layer of tubulin subunits at the ends that still retain their GTP. When this

cap is stochastically lost, the microtubule rapidly depolymerizes. Microtubule assembly and stability, which are self-regulated by the nucleotide state of tubulin, are further modified in the cell by interaction with cellular protein factors (MT-binding proteins), which can stabilize or destabilize microtubules at different points in the cell or at different stages in the cell cycle. Microtubule growth is promoted in a dividing or moving cells and is better controlled in a stable, polarized cells [46].

Microtubules are an essential part of the cytoskeleton. They are involved in almost all major vital processes in eukaryotic cells including cell division, motility, intracellular organelle trafficking and interaction with the environment. Mostly importantly, microtubules are responsible for the organization of the mitotic spindle during cell division. During mitosis MTs are 4-100-fold more dynamic than in interphase [47]. Highly dynamic microtubules are required for the correct attachment of chromosomes, for the transportation of the chromosomes to the metaphase plane, and for the synchronous separation of chromosomes to two daughter cells. Furthermore, microtubules are necessary for cell polarization and the regulation of cellular adhesion during cell migration. The microtubule growth and disassembly locally activate small Rho GTP-ases which regulate actin assembly and contractivity [12]. It was demonstrated that vasicular transport within the cell is facilitated by MTs, and may even be dependent on them. Vesicles were shown to be localized adjacent to microtubular structures and MT-interfering agents can inhibit protein secretion [48] and exocytic responses [49]. The crucial role of microtubules in all these cellular processes, and mainly in cell division, makes them a very attractive target for anti-tumor therapy.

1.3.3 Microtubule stabilizing agents (MTSAs)

Compounds targeting microtubules are one of the most important class of anti-cancer agents. Microtubule-interfering agents are divided in two groups according to their mode of action. Microtubule destabilizers are compounds that prevent the assembly of tubulin heterodimers. Treatment with these compounds leads to the disruption of the MT system in the cell and makes all processes involving MTs (including cell division) impossible. Vincristine, the first MT-interfering agent approved for clinical use, belongs to this group [50]. The two main groups of microtubule stabilizers are taxanes and epothilones (Fig. 1.7) [51]. Over the last half century since MT-stabilizers were discovered, a substantial number of these compounds made there way towards clinical use. Multiple studies on their molecular mechanism of action brought rationales for combination of MT-stabilizers with other treatment modalities in cancer treatment.

Taxanes. Paclitaxel (taxol) was discovered in 1971 as part of an anti-cancer drug screen [52]. It was isolated from an extract of the bark from a rare yew, Taxus brevifolia. In a search for a more abundant supply, docetaxel (taxotere) was developed, which is synthesized from an inactive taxane precursor found in more abundant yew species [51]. Since the 1990s,

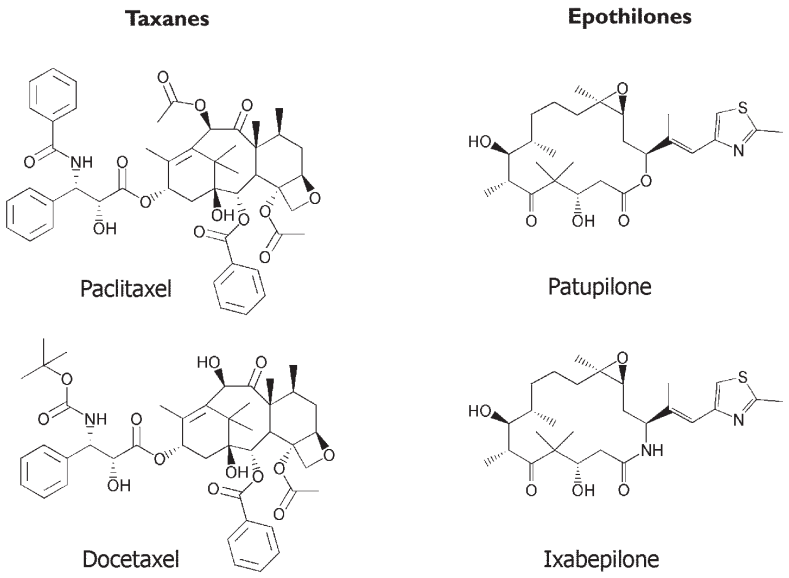

Figure 1.7. Chemical structure of MT-stabilizing agents [51].

paclitaxel and docetaxel were approved by FDA [53-54] for a broad range of indications, including advanced breast cancer [55-56], high-grade ovarian cancer, non-small cell lung cancer [57] and also for a number of other tumors [58].

Epothilones. The epothilones are non-taxoid MT-stabilizers of bacterial origin, which share the binding site on beta-tubulin with taxanes [53, 59-61]. Epothilones are the most clinically promising MT-stabilizing compounds, as they offer a number of advantages: (i) they are very potent MT-stabilizers with cytotoxicity in the low nanomolar concentration range; (ii) they come from an abundant supply and can be obtained by bacterial fermentation; (iii) they are a worse substrate for the P-glycoprotein than taxanes and thus more effective than paclitaxel in cells which acquired multidrug resistance; (iv) due to their relatively simple molecular architecture, epothilone derivatives with more favourable pharmacological profile are available [62]. Different members of the epothilone family are currently in various stages of clinical development as antitumor compounds. Ixabepilone is the first FDA approved drug in this class and indicated for the treatment of patients with metastatic breast cancer. Patupilone is tested in a wide spectrum of phase II studies, including those for recurrent glioblastoma, prostate, cervical, renal cell, gastric and lung tumors [63].

1.3.4 Biological effects of microtubule stabilizing agents

Taxanes and epothilones bind to beta-tubulin in a close proximity to residue Thr274 [64]. Compounds from both groups can induce polymerization of the purified tubulin heterodimers, even under extremely unfavourable conditions and stabilize microtubules under depolymerising conditions [61, 65]. Exposure to epothilones and taxanes prevents shortening of MT resulting in the accumulation of polymerized MT molecules, which then form bundles in the cytoplasm [61, 66-68]. At lower doses of MTSAs, MT bundles formation cannot be observed but a signifi-

cant deceleration of microtubule dynamics does occur [60, 69].

MTSAs activate intracellular signalling pathways and can influence transcription. On the transcriptional level, MTSAs were shown to induce genes responsible for stress response and cell cycle checkpoints [70-71]. On the level of intracellular cell signalling, paclitaxel was shown to induce the PI3K/Akt pathway [72]. Several reports indicate the induction of the NF-kB pathway after treatment with MTSAs [73-75]. There is no clear understanding as to whether these signalling and transcriptional events are a consequence of MT-function inhibition or MT- independent events. Quite likely, it is the former, however, there is a dearth of systemical studies of this problem.

The treatment of cells with MTSAs inhibits cell proliferation. Classical mechanism, which was first discovered for taxanes, is induction of M-phase arrest as a result of MT-bundling and destruction of mitotic spindle [61, 69, 71, 76]. This old dogma that cytotoxic effects of MTSAs are related to the induction of M phase arrest is hardly questioned nowadays. After incubation with low concentrations of MTSAs, aberrant mitosis takes place. This is a consequence of multipolar spindle formation, which results in aneuploid cells, arresting in G1 afterwards [71, 77-78]. It was also shown in human colon carcinoma cells that patupilone is cytotoxic at concentrations that do not induce the M-phase arrest but lead to S phase accumulation [79]. Whatever happens to a cell, be it cell cycle arrest or aberrant mitosis, it will eventually lead to cell death either by classical p53-dependent apoptosis [61, 69, 71] or by alternative mechanisms, like mitotic death [80].

MTSAs can inhibit processes that are necessary for cell invasion. Paclitaxel was shown to inhibit migration of multiple cell types, including endothelial cells [81-83], smooth muscle cells [84] and tumor cells of different origin [85-87]. For example, it reduced the invasive activity of melanoma cells by impairing the secretion of MMP-2/MMP-9 [48]. Patupilone was also shown to inhibit endothelial cell migration [88]. These findings suggest that MTSAs must be effective inhibitors of metastatic processes and, indeed, there are pre-clinical reports in the literature, which support this supposition. When tested on mice, paclitaxel inhibited melanoma metastasis to the lung *in vivo* in a spontaneous melanoma metastases model [89] and in a 4T1 breast cancer model [90]. Patupilone reduced brain metastases in a human prostate xenograft [91] and in a human NSCLC xenograft model in nude mice [92]. Sagopilone (another member of the epothilone family) inhibited breast cancer bone metastasis in breast metastatic mouse model [93].

Due to the strong cytotoxic potency of MTSAs against proliferating cells, they are successfully used as anti-neoplastic drugs nowadays (rev [60]) and their potential anti-metastatic action makes them even more attractive chemotherapeutics for anti-cancer therapy.

1.4 Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

1.4.1 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. They are mosaic proteins, each constituted by a modular combination of inserts and domains (Fig. 1.8). MMP proteins may include (from N- to C-terminus) a signal peptide for secretion, a ~80-residue zymogenic pro-peptide, a ~165-residue zinc- and calcium-dependent catalytic metalloprotein domain and a hemopexin-like domain for collagen binding, pro-MMP activation and dimerization. Gelatinases A (MMP-2) and B (MMP-9) contain additionally a fibronectin-related domain for interaction with collagens and gelatins. Membrane type MMPs (MT-MMPs) also have a trans-membrane domain, a membrane linker and a cytoplasmic tail. In humans, 23 MMPs

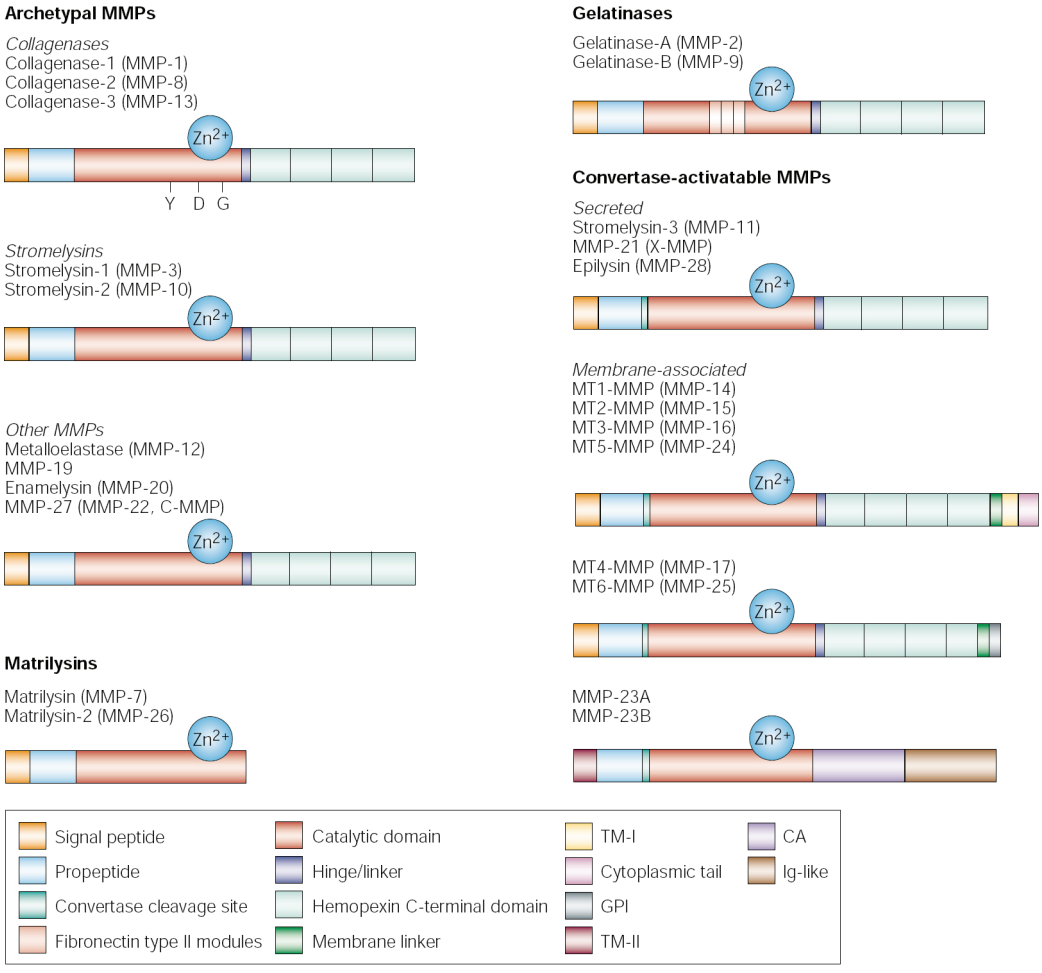

Figure 1.8. Schematic representation of the structure of the 24 human matrix metalloproteinases (MMPs). Y, D, and G represent tyrosine, aspartic acid and glycine amino acids that are present in the catalytic domain of all collagenases. GPI – glycosylphosphatidylinositol membrane anchor, TM – type I or type II transmembrane segments, CA – cysteine array, Ig – immunoglobulin domains [97].

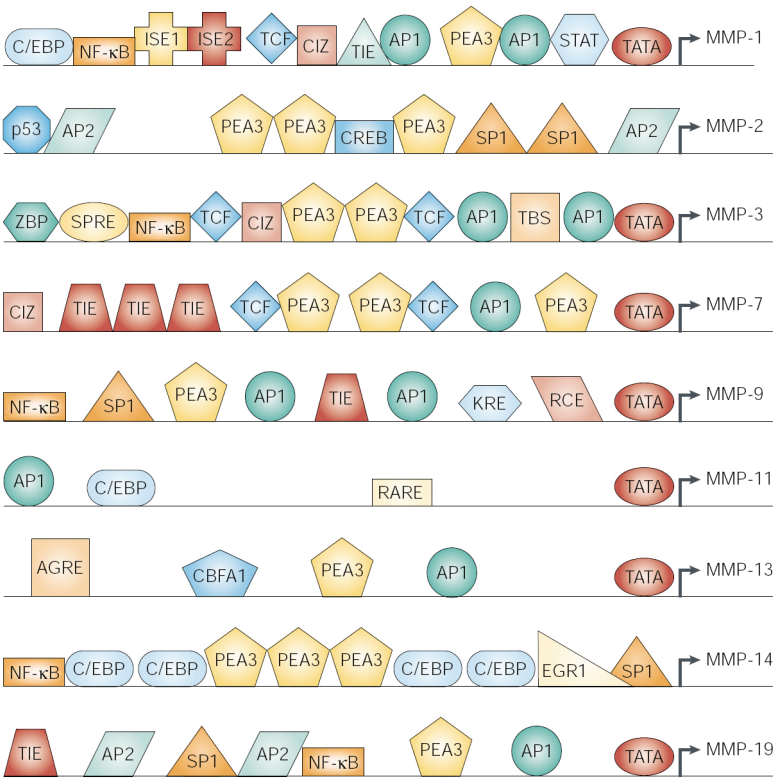

Figure 1.9. Regulatory elements in the promoter regions of human MMP genes. The promoters are shown in the direction 5′-3′, with the transcription start sites indicated with a bent arrow, and the transcription-factor-binding sites placed within boxes. The relative positions of the different elements are not drawn to scale. AGRE – the AG-rich element; AP – the activator proteins-1 and -2 site; CBFA1 – the core-binding factor 1 site; C/EBP – the CCAAT/enhancer-binding protein site; CIZ – the CAS-interacting zinc-finger protein site; CREB – the cyclic AMP response-element binding protein site; EGR1 – the early growth response-1 site; ISE – the immortalization-sensitive elements-1, and -2; KRE – the keratinocyte differentiation-factor responsive element; NF-κB – the nuclear factor of κB site; PEA3 – the polyomavirus enhancer-A binding-protein-3 site; RARE – the retinoic-acid response element; RCE – the retinoblastoma control element; SPRE – the stromelysin-1 platelet-derived growth factor-β responsive element; STAT – the signal transducer and activator of transcription site; TATA – the TATA-box; TBS – the TEL (translocation-ETS-leukaemia) binding site; TCF – the T-cell factor site; TIE – the transforming growth factor-β inhibitory element site; ZBP-89 –– the binding site for 89-kDa zinc-binding protein [97].

were described; these are numbered 1 to 3, 7 to 17, 19 to 21 and 23 to 28 for historical reasons. They can be divided into the following categories based on their preferable substrates: (i) true collagenases (MMP-1, -8, -13), which cut triple-helical collagen at a single site across the three chains; (ii) gelatinases (MMP-2, -9), which target denatured collagens and gelatins; and (iii) stromelysins (MMP-3, -10, -11), which have broad specificity and may degrade proteoglycans. MMPs overlap substantially in substrate specificity and in cellular localization and this complicates investigation of their biological functions [94-95].

Regulation of MMPs. MMPs are regulated via modulation of their gene expression, compartmentalization and inhibition by protein inhibitors. The promoter regions of MMPs contain multiple transcription factor-binding sites (Fig. 1.9), for example the activator proteins (AP)-1 and -2 sites, the nuclear factor kB (NF-kB) site, the retinoblastoma control element (RCE), the TATA-box (TATA) [96-97] etc. Therefore, the transcription of MMPs can be variously regulated by different intra- and extracellular signalling. For example, increased

activation of the WNT pathway increases the expression of MMP-2, -7, -9 and MMP-14 (MT1-MMP) as their promoters contain putative TCF-binding sites [98-99]. PI3K/Akt and p38/Akt pathways were shown to upregulate MMP-2 production in glioma cells after IR [100]. IL-17 stimulates MMP-1 expression in primary human cardiac fibroblasts via p38 MAPK- and ERK1/2-dependent NF-kB and AP-1 activation [101]. The NF-kB pathway activation was also shown to induce MMP-9 [102] and MMP-3 [103].

MMPs are expressed as inactive zymogens. Their proteolytic site is shielded by a pro-domain which must be cleaved for zymogen activation. About one-third of MMPs, including all membrane-bound MMPs, are activated in the intracellular compartment. These MMPs have the convertase cleavage sites (RXKR or RRKR), which are target for pro-protein convertases or furins (serine proteases present in the Golgi network) [104]. For many other MMPs, the mechanism of activation is more presumed than proved. In *ex vivo* systems it was shown that MMPs can be activated by other MMPs [105] and plasmin [106]. However, there is no clear proof that this is also the case *in vivo* [107]. The only well described mechanism is the activation of proMMP-2 by MMP-14 (MT-MMP-1) in the presence of the tissue inhibitor of MMP-2 (TIMP-2) [108], which will be discussed in more detail below (Chapter 1.4.4).

The third level of MMP regulation is the inhibition of their enzymatic activity. There are several groups of natural MMP inhibitors. α2-macroglobulins (approximately 700 kDa glycoproteins) non-specifically inhibit endopeptidase activity in the plasma. They trap MMPs and the complexes of macroglobulins, and the complexes are cleared from plasma by endocytosis [96, 108-109]. Tissue inhibitors of MMPs (TIMPs) are a family of very potent inhibitors, which will be described in detail below (Chapter 1.4.3). Recently another inhibitor of MMPs was described – RECK (reversion-inducing-cysteine-rich protein with Kazal motifs) [110]. This transmembrane glycoprotein regulates at least three members of the MMP family: MMP-2, MMP-9 and MMP-14. This inhibitor seems to be of a particular interest as it is present on the membranes of normal cells and lost during malignant transformation [111].

The last potential mechanism of MMP regulation, which has emerged only recently, is sub-cellular compartmentalization. It was shown that many secreted MMPs are not only released to the extracellular space but also remain associated with the cell membrane via integrins or other cellular receptors [107]. This allows to maintain a high density of proteases in the pericellular space. Endocytosis was also shown as a mechanism for MT1-MMP regulation [112]. Additionally, MMPs are secreted at least partially in vesicles [113-114], which allows for an additional potential mechanism of MMP regulation based on the MT and actin cytoskeleton.

1.4.2 Physiological role of MMPs

MMPs were discovered as enzymes that degrade structural components of the extracellular matrix (ECM) for cell invasion. However during the ECM degradation specific fragments

with independent biological activities can be produced. For example, the cleavage of laminin-5 or collagen IV molecules results in the exposure of cryptic sites, which promote migration via integrin signaling. MMPs can also modify the activity of signaling molecules, as cleavage of ECM proteins can release ECM-bound growth factors, including insulin growth factor (IGF) and fibroblast growth factor (FGR) [115]. In recent years, a number of non-matrix substrates of MMPs were identified. Precursors of molecules that alter cell growth are activated by MMP-mediated cleavage, such as IL1-β and insulin-like growth factor binding protein which is cleaved into active insulin growth factor ligand. MMP-7 was shown to participate in regulation of apoptosis by cleaving membrane-bound Fas ligand [116]. A role of MMP in intercellular communication has also been demonstrated. For example, MMP-9 is required for VEGF release for osteoblast recruitment during bone development [117].

By implication of genetic knock-outs, the functions of MMPs were linked to many vital biological processes, (e.g. embryonic development, organ morphogenesis, ovulation and endometrial cycling, hair follicle cycling, bone remodeling, wound healing, angiogenesis and apoptosis). If the enzymatic activity of MMPs is not subjected to a fine-tuned control, dysregulation can give rise to pathogenic activity. This occurs during inflammation, arthritis, fibrosis and cancer-associated processes [115, 118].

1.4.3 Tissue inhibitors of MMPs (TIMPs)

The tissue inhibitor of MMPs (TIMP) family consists of four members, TIMP-1 to -4, which are substantially conserved throughout the evolution [119]. Members of the TIMP family share sequence homology [120] and about 40 % structural identity at the protein level. TIMPs have two structurally similar domains: an N-terminal domain with six conserved cysteine residues possessing MMP-inhibitory activity, and a C-terminal domain, which also contains six conserved cysteine residues [121]. All members of the family are secreted proteins. TIMP-3 is noted for its ability to bind to the ECM [122].

Regulation of TIMPs. Whereas TIMP-2 is constitutive and widely expressed throughout the body, TIMP-1, -3, -4 expression is inducible and often exhibits tissue specificity. TIMP-1 is enriched in reproductive organ systems, TIMP-3 in heart, kidney, and thymus, and TIMP-4 in brain, heart, ovary and skeletal muscle [123]. The regulation of TIMPs is not well studied. The upregulation occurs primarily at the transcriptional level involving transcription factors like AP-1 (TIMP-1, -2, -4), SP-1 (all TIMPs) and NF-kB (TIMP-2 and -4) transcription factors. The presence of an SP1 site in all TIMP promoters reflects the involvement of TIMPs in developmental processes. Tissue-specific promoter binding sites can be also found; for example, the promoter of TIMP-4 contains a myogenin-binding site, likely important for its skeletal muscle-specific expression [124].

Since the common promoter elements can be found both in MMPs and TIMPs (e.g. AP-1, NF-kB) these proteins can be synchronously induced by extracellular stimuli, like growth

factors, cytokines (IL-1β) or phorbol ester (activator of PKC) [125-126]. However, an increasing amount of evidence suggests the existence of signalling pathways that can lead to TIMP-1 upregulation without MMP induction. For instance, erythropoietin regulates TIMP-1 and MMP-9 expression in an inversely coordinated manner [127] and the transforming growth factor-β1 (TGF-β1) upregulates TIMP-1 and downregulates MMP-1 by differentially utilizing the AP1 and Smad pathways [128].

1.4.4 Physiological role of TIMPs

The first known biological function of TIMPs, which led to their discovery, was the inhibition of MMPs. In the ECM, TIMPs form non-covalent 1:1 stoichiometric complexes with MMPs. Almost all MMPs can be inhibited by all 4 TIMPs, although differences in binding affinity have been reported. For example, it is known that TIMP-1 is a weak inhibitor for MT-MMPs [129]. The mechanism of inhibition is believed to be the following: TIMP molecules have a wedge-like shape, which fits into the active site cleft of an MMP, as would a substrate molecule. The conserved cysteine in the N-terminal domain, at least in TIMP-1, will subsequently chelate the active zinc site thereby inactivating the MMP protein [120, 130].

Except MMP inhibition, TIMPs are also required for MMP activation. The process of proMMP-2 activation is well described. MTl-MMP (MMP-14) activates proMMP-2 by cleaving its pro-domain and TIMP-2 serves as an adaptor protein between the two MMPs. TIMP-2 mediates proMMP-2 activation in a dose-dependent manner. A certain ratio of TIMP-2 and proMMP-2 must be maintained for optimum activation [131]. The proMMP-TIMP complex is formed between the C-terminal domain of the inhibitor and the C-terminal hemopexin domain of proMMP, thus both molecules maintain their proteolytic and inhibitory properties, respectively. These non-inhibitory complexes between pro-gelatinases and TIMPs are restricted to proMMP-2 and TIMP-2, -3, or -4 on the one hand and to MMP-9 and TIMP-1 on the other. The significance of the latter complex is not clear, it could possibly play a role in proMMP-9 activation, by analogy with the TIMP-2/MMP-2 case [108, 120, 132-134].

It is difficult to distinguish between biological functions of TIMPs that are fulfilled through MMP-related mechanisms (e.g. regulation of angiogenesis, cell growth and apoptosis) and their unique MMP-independent functions. In an attempt to differentiate between these functions, studies were performed involving small molecule MMP-inhibitors and TIMP mutants without MMP-binding activity. These studies could demonstrate that, independently of the MMP-inhibiting activity, TIMP-1 and TIMP-2 can promote growth of a wide range of cells, including cells of the erythroid lineage, smooth-muscle cells, keratinocytes, fibroblast and epithelial cells. The mitogenic signals of TIMPs are probably transduced through tyrosine-kinase receptor pathways; however, no receptors for TIMPs have been found thus far [122, 135-136]. Other MMP-independent functions are the anti-apoptotic effect of TIMP-1, demonstrated in Burkitt's lymphoma cell lines [137] and the anti-angiogenic effect of TIMP-2

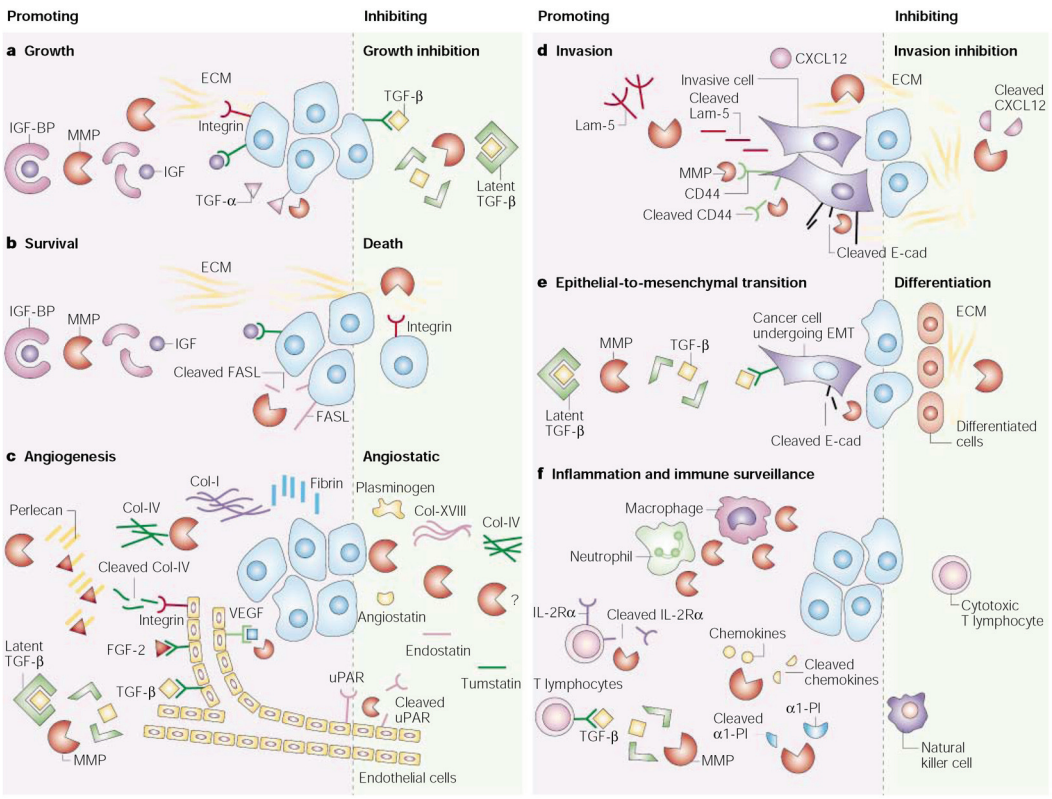

Figure 1.10. Promoting and inhibiting functions of MMPs in cancer progression [139].

[138]. Taken together, TIMPs have a broad range of biological functions. The first set of functions is related to MMP activity-regulated processes, but the second considerable part is unrelated to MMPs and extended to direct cellular effects.

1.4.5 Role of MMPs and TIMPs in cancer progression

The expression of TIMPs, MMPs and MMPs activity are increased in almost every type of human cancer. The expression rate positively correlates with an advanced tumor stage, increased metastasis and shortened survival [139-142]. Unlike classical oncogenes, MMPs and TIMPs are not upregulated by gene amplification or activating mutations. Their increased expression in tumors is most likely due to transcriptional changes, which can be a result of activated oncogenes (e.g. Ras) [143] or loss of tumor suppressors (for example p53) [144] which are involved in the regulation of MMP expression. In human tumors, cancer cells are not the only source of MMPs that can be also synthesized by stromal cells. MMPs and TIMPs, as their inhibitors, are involved in several steps of cancer development and paradoxically implicated in both cancer promotion and inhibition. The pathways leading to opposing effects on cancer progression are sometimes initiated by cleavage of the same MMP substrate [139].

The diversity of MMP functions in cancer is illustrated in Figure 1.10. An example of a controversial effect of MMPs on tumor growth is the regulation of the transforming growth

factor-β (TGF-β) pathway. TGF-β normally exerts tumor-suppressive effects by enforcing cytostasis and differentiation. This factor can be proteolytically activated by MMPs. However, since the tumor cells often acquire non-responsiveness to TGF-β, the proteolytic activation of TGF-β by MMPs may have tumor-promoting effects by selectively driving stroma-mediated invasion. MMPs promote the survival of cancer cells also by liberating IGF and cleaving FAS death receptor ligand (FASL). MMPs can also indirectly promote apoptosis by changing the ECM composition, which influences integrin signalling (Fig 1.10, b). MMPs promote angiogenesis not only by promoting endothelial cell invasion but also by increasing the bioavailability of the pro-angiogenic growth factors, like VEGF. MMPs also inhibit angiogenesis through the generation of the anti-angiogenic factors angiostatin and endostatin (Fig. 1.10, c). MMPs positively regulate invasion by degrading structural ECM components and by cleavage of adhesion molecules and E-cadherin. MMPs have the potential to inhibit metastasis, as they cleave SDF-1, a chemokine of the CXC family that promotes breast cancer metastasis (Fig. 1.10, d). MMPs promote the epithelial-to-mesenchymal transition (EMT) – a process, which is associated with cell dedifferentiation during malignant transformation – by cleaving the cell-adhesion molecule E-cadherin and by liberating TGF-β. MMP-9 can also promote differentiation by mechanism that is thus far unknown [139] (Fig. 1.10, e). MMPs are involved in immune reactions against cancer cells. For example, MMP cleavage of the IL-2 receptor-α (IL-2Rα) on T lymphocytes results in inhibition of the T-lymphocyte proliferation (reviewed in [132, 139]).

Over the past few years, it has become clear that MMPs do more than just promote invasion and metastasis by degradation of the ECM proteins. The opposing functions of MMPs make them simultaneously act as tumor promoting and inhibiting factors, but most of the literature supports the opinion that the sum of MMP actions promotes cancer progression. MMP inhibitors are potential contributors to cancer treatment; however, such inhibitors for neoplastic treatment must be used with care, as the MMP functions that inhibit cancer progression must be taken into consideration.

1.5 Combined treatment modality of MTSAs and IR: radioenhancing mechanisms

The clinical objective of combining drugs with radiation is to improve survival without compromising life quality of patients. Much of clinical and experimental work on the combined treatment modalities to date has been empirically rather than rationally driven. With the recent emergence of knowledge in molecular oncology, new combination therapy strategies are being proposed that better capitalize on mechanisms of drug-radiation interactions. In their article Bentzen et al [145] proposed five mechanisms for a rational design and analysis of drug-radiation therapies: (i) spatial cooperation – a strategy employing combined treatment to target locoregional and systemic disease; (ii) cytotoxic enhancement – increased

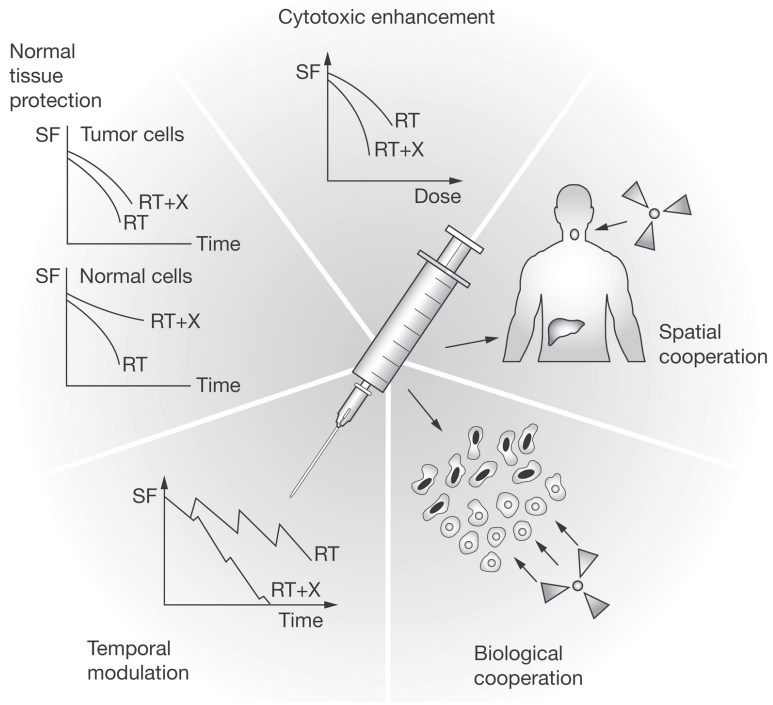

Figure 1.11. Representation of the interplay between spatial cooperation, cytotoxic enhancement, biological cooperation, temporal modulation and normal tissue protection. RT – radiotherapy, SF - surviving fraction, X – drug [145].

cell killing by modulating repair of cellular DNA damage; (iii) biological cooperation – targeting distinct cell populations or employing different mechanisms for cell killing; (iv) temporal modulation – enhancing tumor response to fractionated radiotherapy and (v) normal tissue protection (Figure 1.11).

The combination of MTSAs with IR involves direct drug-radiation interactions at the cellular level – cytotoxic enhancement, biological cooperation and temporal modulation. These strategies primarily aim to interfere with repair mechanisms and target distinct cell populations (e.g. hypoxic cells) and structures of the tumor microenvironment (vasculature).

1.5.1 Cell cycle specific radioenhancing mechanisms (cytotoxic enhancement)

It is known already for decades that radio-sensitivity of cells differs between cell cycle phases, that mitotic cells are the most radiosensitive and cell during S phase are the most resistant [20]. The first study to demonstrate that paclitaxel radio-sensitizes human astrocytoma cells to IR [146] was performed ten years after it became known that paclitaxel induces accumulation of cells in M-phase [68]. Since then, several *in vitro* studies have demonstrated that taxanes enhance irradiation sensitivity by the accumulation of cells in the G2/M phase of the cell cycle [147-150]. Upon further investigations, it was shown that the radioenhancing mechanism based on the G2/M-block was dominant in paclitaxel-resistant tumors, where the arrested cells were not doomed to cell death by drug treatment alone and the M-phase arrest enhanced the cell-cycle specific sensitivity to subsequent IR [151-152]. Epothilones were also

probed for radiosensitizing properties. The semisynthetic epothilone B (BMS-247550) was evaluated in *in vivo* and *in vitro* settings for radioenhancing effects in human lung cancer cells and the mechanism of observed radioenhancement was attributed to the G2/M phase arrest [153].

The classical M-phase arrest-related mechanism of radio-sensitization by MTSAs was opposed by a study with patupilone performed by Hofstetter and co-workers [79]. In this study a very strong radiosensitizing effect of patupilone was observed at concentrations of the compound that induced not an M-phase arrest but accumulation in S phase instead. The authors speculated that a decelerated S phase transgression-related mechanisms could be responsible for the observed radiosensitization. Interestingly, the radiation potentiating effect was also seen in non-proliferating ovarian carcinoma cell lines [150], again suggesting the existence of radioenhancing mechanisms different from the widely excepted M-phase-related radiosensitivity.

1.5.2 Reoxygenation radioenhancing mechanisms (biological cooperation and temporal modulation)

The "tumor cell reoxygenation" theory (named by analogy with a classical radiobiological terminology) arose in an attempt to explain the time dependent radioenhancing effect of paclitaxel that was not correlating with M-phase accumulation of tumor cells [154]. The essence of the theory is that after exposure to the drug, the tumor oxygenation increases, which results in increased radiosensitivity. The tumor "reoxygenation" occurs as follows: after being treated with paclitaxel, the tumor experiences a massive cell loss due to apoptosis or necrosis [151, 155]. The cell loss reduces the overall oxygen consumption rate and previously hypoxic cells receive a better access to oxygen. The reoxygenation theory was supported by studies of Milas and co-workers, where they showed that paclitaxel had radiosensitizing effect on murin mammary carcinoma tumors only under air-breathing conditions but not under hypoxic conditions. In theses tumors, the pO2 was increased after paclitaxel treatment [156]. The "reoxygenation"-related radiation enhancement takes place in drug-sensitive cell lines, whereas M phase-related mechanism occurs more prevalently in resistant cells or at lower doses of MTSAs [152].

1.5.3 Tumor microenvironment-related radioenhancing mechanisms (biological cooperation and temporal modulation)

The great potential of tumor microenvironment-directed properties of MTSAs has only recently started to emerge, reflecting a better understanding of the importance of the tumor stroma for the treatment outcome. The studies that showed a higher radiosensitizing ability of patupilone *in vivo* than *in vitro* cell systems [79, 157]; they also indicated that the tumor

response to the combined treatment occurs not solely on the level of tumor cells.

Anti-angiogenic properties of MTSAs. As endothelial damage is fundamental for tumor control, the radioenhancement by MTSAs may be due to their effect on the tumor endothelial system [158]. The analysis of the emerging data shows that MTSAs can have both a direct and an indirect effect on tumor angiogenesis [159]. The direct effect can be attributed to inhibition of proliferation of endothelial cells and decrease of endothelial cell migration and capillary-like tube formation *in vitro* after treatment with MTSAs [88, 160-163].

The indirect anti-angiogenic effect is mediated by tumor cells, when the anti-neoplastic effect of MTSAs leads to depletion of tumor cell-secreted pro-angiogenic factors. This may happen by way of different mechanisms, including simple tumor cell loss, suppression of VEGF expression or secretion [164-165] and inhibition of HIF-1 expression and the consequent decrease in HIF-dependent protein expression [166]. There is strong experimental evidence to suggest that the indirect effect on tumor vasculature is the most prevalent one. A decrease in microvessel density (MVD) after treatment with patupilone was only observed in patupilone-sensitive lung tumor xenografts but not in xenografts originated from patupilone-resistant cells [157]. Moreover, the inhibition of HIF-1-dependent expression after patupilone treatment was only observed in drug-sensitive human ovarian cancer cells but not in their resistant counterparts [166].

The anti-angiogenic effect of MTSAs translates in the destruction of blood vessels and reduced MVD [157, 162, 167]. The decrease in MVD should lead to an increase of hypoxic regions within the tumor, which would reduce ability of IR to kill the tumor cells. However, the radioenhancement by MTSAs may be explained by a phenomenon known as “vasculature normalization” after anti-angiogenic treatment [168]. This effect is characterized by loss of only immature, non-functional vessels in the tumor, which improves oxygenation due to a better perfusion of the remaining vasculature. As a result of vascular normalization, the decrease of MVD negatively correlates with tumor oxygenation [169]. However, it was demonstrated that the radioenhancing effect of the patupilone in lung tumor xenografts in mice can also be seen on the background of the compound-induced hypoxia in the tumor [157]. Patupilone is an equally potent anti-proliferative agent against normoxic and hypoxic cells. Therefore, the supra-additive anti-tumor effect of patupilone in this case may be a result of cooperative action of multiple processes, including cell kill by radiation, by drug and by reduced oxygen and nutrition supply due to MVD reduction.

Anti-metastatic properties of MTSAs. The potential role of radiotherapy in the induction of metastases was already discussed in detail above (see Chapter 1.2.2). In short, IR was shown to promote cell invasion *in vitro* and metastasis *in vivo* in mouse models [28-33]. This potentiating effect is most probably attributed to the ability of IR to induce the expression of MMPs (secreted proteinases that are required for cell invasion) [100, 102, 170-171]. The importance of MMPs for cancer progression and treatment response has only recently arisen

and has not yet been properly addressed in clinical research. However, there is increasing evidence that MMPs in tissue and plasma of cancer patients are upregulated after irradiation [142, 172].

MTSAs are very promising anti-metastatic agents. Multiple studies demonstrated that taxanes and epothilones can reduce migration of different cell types: smooth muscle cells [83] endothelial [81, 83, 88] and tumor cells (for example, ovarian and colon carcinoma) [86-87]. The anti-metastatic properties of paclitaxel and epothilones were also demonstrated in tumor-bearing mice (for lung, prostate and breast tumors) [90-91, 93, 173].

The secretion of MMPs and TIMPs can also be targeted by MTSAs, as they are at least partially dependent on the dynamic functional MT system [113-114]. At low doses, paclitaxel impaires the secretion of MMP-2 and MMP-9 by human melanoma and prostate cancer cells, inhibiting cell invasion [48, 174]. One has to keep in mind, however, that the effect of MTSAs on MMP expression may be differential as paclitaxel enhanced MMP-2 and -9 expression in murine and human tumor cells, resulting in increased cell invasion [175-176].

MTSAs showed potency as anti-metastatic drugs in pre-clinical studies. These findings represent an additional rational for combining MTSAs with IR for cancer treatment. IR can induce MMP activity and this has to be taken in consideration during the radiation therapy regardless of metastasis induction risk, as during the last decade strong evidence arises that MMPs play an important role in cancer progression, not only for tumor dissemination but also in regulation of angiogenesis, cell survival etc. (see Chapter 1.4.3). MTSAs have a strong potential to inhibit cell invasion and MMP function, therefore a combination of these compounds and IR for cancer treatment might be of great benefit.

1.6 Aim of the study

The combined application of microtubule stabilizing agents (MTSAs) with ionizing radiation is a highly promising treatment modality in cancer therapy [79, 153, 157]. Our own laboratory investigated the anti-tumor activity of the novel, clinically relevant MTSA patupilone (epothilone B). A strong supra-additive effect on the growth of several types of tumor xenografts in mice was demonstrated when patupilone was administered in combination with IR [79]. Interestingly, the additive cytotoxic effect of patupilone and IR was much less pronounced *in vitro*, indicating that an additional mechanism on the level of the tumor microenvironment (TME) may contribute to the supra-additive *in vivo* response. Further investigations revealed that this supra-additive anti-tumor effect of patupilone and IR *in vivo* can only be observed in tumors derived from patupilone-sensitive cancer cells [157]. This suggests that the effect on the TME may be an indirect effect mediated by cancer cells, perhaps via the secretion of active molecules. Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are important components of the TME, which are secreted both by tumor-associated stromal cells and by cancer cells. The overexpression of MMPs and TIMPs was reported for almost all tumor types and associated with inferior survival prognosis [132, 139]. Furthermore, MMPs are upregulated after IR and can contribute to IR-induced tumor aggressiveness [33]. As secretion of MMPs at least partially depends on the microtubule system [48, 113], MTSAs are potential inhibitors of MMP functions.

The goal of the present study was to investigate *in vitro* whether the MTSA patupilone influences the function of MMPs in IR-treated human tumor cells. To approach the question we defined the following aims: 1) to assess the changes in the activity of secreted MMPs after irradiation and the effect of patupilone treatment on these changes; 2) to evaluate the biological relevance of the treatment-altered MMP activity in functional cell invasion assay; 3) to investigate the mechanisms underlying the observed MMP activity alterations on different levels of the MMP regulatory cascade.

2 Materials and Methods

2.1 Cell lines and preparation of conditioned cell culture media

All cells (Table 2.1) were grown at 37° C in a 5 % CO_2 humidified atmosphere. Conditioned cell culture media (CM) was prepared as follows: after indicated treatment cells were maintained in serum-free Ultraculture medium (Lonza, Fischer Scientific) for 24 h. The cell culture medium was collected, centrifuged at 10'000x g for 5 min at 4° C and the supernatants were stored at -80° C. CM from HCT116 and U251 cells were concentrated using Amicon Ultra centrifuge concentrators Ultracel-5k (Millipore) 10-15-fold and 4-5-fold, respectively.

Table 2.1. Human cell lines and cell culture media.

Cell line	Description	Basal medium	Additives
HT1080 ATCC No CCL-121	fibrosarcoma cells, (a gift of Prof. B. Odermatt, University of Zurich)	IMDM	10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin-streptomycin
HCT116 ATCC No CCL-247	Human colon carcinoma cells	RPMI	10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin-streptomycin and 1 % (v/v) L-glutamine.
U251	glioma cells, (a gift of Prof. K.Frei University of Zurich)	DMEM	
DAOY ATCC No HTB-186	medulloblastoma cells	Improved MEM	10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin-streptomycin
D341 ATCC No HTB-187	medulloblastoma cells	Improved MEM	10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin-streptomycin, 1 % NAAA
D425	medulloblastoma cells	Improved MEM	

2.2 Reagents and Antibodies

Reagents and Antibodies (ABs)	Manufacturer
Alamar Blue	Biosource Int.
All cell culture media and supplements	Gibco, Invitrogen
anti-MMP-14 AB, rabbit polyclonal	Abcam
anti-MMP-3 AB, mouse monoclonal	Santa Cruz Biotechnology, Inc.
anti-MMP-9 AB, rabbit monoclonal	Abcam
anti-TIMP-1 AB, neutralizing, goat	R&D Systems
anti-TIMP-2 AB, neutralizing, goat	R&D Systems
anti-β-actine AB, mouse monoclonal	Sigma
anti-β-tubulin AB, mouse monoclonal	BD Pharmigen
APMA (4-aminophenylmercuric acetate)	AnaSpec
Control goat IgG	Sigma
Fast SYBR® Green Master Mix	Applied Biosystems
LipofectamineTM 2000	Invitrogen
Matrigel™	BD
NNGH (N-Isobutyl-N-(4-methoxyphenylsulfonyl) glycyl hydroxamic acid)	Calbiochem®
ProLong® Gold Antifade Reagent	Invitrogen
RNAse A	Quigen
secondary anti-goat AB, donkey, HRP-linked	Santa Cruz Biotechnology, Inc.
secondary anti-mouse AB, from goat, AlexaFluor®488-linked	Molecular Probes®, Invitrogen
secondary anti-mouse AB, from sheep, HRP-linked	GE Helthcare
secondary anti-rabbit AB, from donkey, HRP-linked	GE Helthcare
SensoLyte™ 520 Generic MMP-2 assay kit	AnaSpec
All other chemical reagents	Sigma-Aldrich®

2.3 Irradiation and Patupilone

Irradiation was performed at room temperature using a Gulmay Xstrahl 200 kV X-ray unit at 1 Gy/min. Patupilone (epothilone B, EPO906) was provided by Novartis Pharma AG (Basel, Switzerland). Patupilone was dissolved in DMSO as 1 mM stock solution and stored at -20° C. For the experiments, it was further diluted in the corresponding cell culture media. For each experiment, fresh dilutions were prepared.

2.4 Proliferation assay

Cell proliferation was assessed using the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT)-like Alamar Blue (Biosource Int.) colorimetric assay according to manufacture's instructions. Absorption at 570/670 nm was measured with an Ultra Microplate Recorder (Bio-Tech Instruments Inc.). Cells were seeded in 96-well plates and treated with increasing concentrations of patupilone 24 h prior to irradiation with increasing doses of IR. Proliferative activity was measured daily for 72 h after IR. All measurements were performed in triplicate in at least 3 independent experiments.

2.5 Clonogenic assay

The amount of plated cells was adjusted to obtain ~100 colonies per cell culture dish with a given treatment. 24 hours after plating cells were treated with increasing concentrations of patupilone and irradiated 24 h later. Colonies were allowed to grow under normal cell culture conditions for 7-10 days before fixation with Methanol/Acetic acid (75 %/25 %, v/v) and staining with 2% crystal violet. Colonies with more than 50 cells were counted manually. The surviving fraction (SF) with a given treatment was determined as follows: SF = (amount of counted colonies) / (amount of seeded cells x PE), where PE is a plating efficiency of untreated cells: PE = (amount of counted colonies) / (amount of seeded cells). For the determination of the synergistic effect, the earlier described model of synergy was used [177] and the following equation was applied: D = (SF(treatment 1) x SF(treatment 2)) / SF(treatment 1+2). When D > 1, treatments were considered to have a synergistic effect, when D = 1 – an additive effect and D < 1 – a less than additive effect.

2.6 Cell cycle distribution analysis

Cell cycle distribution was determined by analysis of DNA content in cells by Flow cytometry. Cells were grown under normal cell culture conditions with different treatments applied. Then cells were trypsinized, washed with PBS and fixed with 70 % ethanol and stained with propidium iodide (50 µg/mL, containing 14 kU/mL RNAse A). The cells were analyzed on FACS Canto flow cytometer (BD Biosciences). Cell cycle analysis was performed using FlowJo 7 software (Tree Star Inc) by applying Dean-Jett-Fox model.

2.7 MMP activity assay

MMP activity in non-activated CM was determined using a FRET-based MMP-2 activity assay (SensoLyte MMP-2 assay kit, AnaSpec). Even though – according to the kit de-

scription – this assay is designed for the measurement of specific MMP-2 activity, the used peptide substrate can be cleaved by multiple MMPs in CM. Therefore we did not consider it to be specific for MMP-2 and used it for the determination of pooled MMP activity. The substrate cleavage reaction was performed according to the manufacture's instructions. In order to determine membrane-bound MMP activity, cells were washed twice with fresh serum-free media and incubated with the FRET assay substrate. The fluorescent signal was measured in a Tecan GENios spectrophotometer at 31° C every hour for 14 hours. The acquired signal was adjusted to the cell number and presented as the initial velocity of the substrate cleavage reaction (V0), relative to control. V0 was determined as a slope of the curve – the fluorescent signal plotted versus time – using linear regression (GraphPad Software, Inc).

2.8 Cell migration assay

Transwell inserts (6.5 mm, 8 µm pores, Costar) were either coated with Matrigel™ (BD) for the cell invasion assay or left uncoated for the cell motility assay. Complete cell culture media with 10 % FCS was used as an attractant for migrating cells. For HCT116 cells, the attractant was a mixture of complete cell culture medium (with 10 % FCS and penicillin/streptomycin) and 48 h conditioned media from HCT1116 (5:1) cells. 5'000 cells/insert for HT1080 cells, 8'000 cells/insert for U251 cell and 50'000 cell/insert for HCT116 cells were seeded in 200 µL of serum-free media. Patupilone was added to cells at the moment of seeding and cells were irradiated 4 h thereafter. Cells were allowed to migrate for 24 h. For quantification, cells from the upper side of the insert were scraped away; and inserts were fixed in Methanol/Acetic acid (75 %/25 %, v/v) and stained with DAPI. The invaded cells were counted manually under the microscope or using the software (Imaris, Bitplane). Each experiment was carried out in quadruplicates.

Coating with Matrigel (BD) was performed as follows: 50 µL of Matrigel, diluted 1:20 with DMEM cell culture medium, was added per insert and dried overnight at RT under sterile flow. On the day of experiment, inserts were rehydrated with serum-free media for 4 h at 37° C.

2.9 qRT-PCR

Total mRNA was isolated with RNeasy Plus Mini Kit (Qiagen). Reverse transcriptase reaction was conducted with 1 µg of mRNA with High capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) on 7900H PCR Instrument (Applied Biosystems). Data was analysed using RQ-manager software (Applied Biosystems), employing the ΔΔCt method. The primers were a generous gift from Professor S. Gay (University Hospital Zurich).

	Primers 5'-3'
MMP-1	F: TGT GGA CCA TGC CAT TGA GAA R: TCT GCT TGA CCC TCA GAG ACC
MMP-2	F: CTT CCG TCT GTC CCA GGA T R: CCC CAT AGA GCT CCT GAA TG
MMP-3	F: GGG CCA TCA GAG GAA ATG R: CAC GGT TGG AGG GAA ACC TA
MMP-9	F: GGC CAC TAC TGT GCC TTT GAG R: GAT GGC GTC GAA GAT GTT CAC
MMP-14	F: TGG AGG AGA CAC CCA CTT TGA R: GCC ACC AGG AAG ATG TCA TTT C
TIMP-1	F: AGT GGC ACT CAT TGC TTG TG R: TTT TCA GAG CCT TGG AGG AG
TIMP-2	F: TTT TGC AAT GCA GAT GTA GTG AT R: TCC TTC TCA CTG ACC GCT TT
TIMP-3	F: ACG CTG GTC TAC ACC ATC AAG R: TTG GTG AAG CCT CGG TAC A
18srRNA	F: ATG GCC GTT CTT AGT TGG TG R: CGC TGA GCC AGT CAG TGT AG
TBP	F: CGG CTG TTT AAC TTC GCT TC R: TTC TTG GCA AAC CAG AAA CC

2.10 Western Blot

The cell lysates were collected by suspending cells in the Laemmli buffer and CM was mixed with 5x Laemmli buffer. Samples were boiled at 95° C for 5 min and subjected to 12 % SDS-PAGE, followed by blotting onto PVDF-membranes using a semi-dry system (Hoefer). Detection was performed using a chemiluminescence detection system (ECL Western Blotting detection system, Amersham, GE Healthcare). Western blots were quantified by Quantity One 4.6 software (Bio-Rad Laboratories). The following antibodies were used: anti-TIMP-1 and TIMP-2 (neutralizing antibodies, R&D systems), anti-MMP-3 (SantaCruz Biotechnology, INC.), anti-MMP-9 and -14 (Abcam) and anti-β-actin (Sigma).

2.11 Gelatinolytic zymography assay

For the determination of gelatinolytic activity, CM samples were mixed with 5x Laemmli buffer without β-mercaptoethanol and loaded on 10 % SDS-PAGE, containing 1 mg/mL gelatin (running conditions: 20 mA per gel, 2.5 h, at 4° C). Then, the gels were washed in 2 % Triton X-100 and incubated in TCN buffer (Tris HCl pH 7.4, 50 mM, NaCl 0.2 M, $CaCl_2$ 5 mM) for 36 h. Gels were stained with 0.2 % Coomassie Blue until transparent bands could be visualized against a dark blue background.

2.12 MMP antibody array

Protein levels of MMP-1, -2, -3, -8, -9, -13 and TIMP-1, -2, -4 were determined in CM using an anti-Human MMP antibody array (RayBiothec). The antibody array was performed according to the manufacturer's instructions. The array membranes were developed using an Amersham western blot film and developed films were scanned and quantified with QuantityOne quantification software (BioRad Laboratories). Values were adjusted to the cell amount and presented relatively to the control. At least two independent experiments were performed for each treatment condition.

2.13 Transfection with siRNA and cDNA constructs

Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNAs for downregulation of human TIMP-1 (GenBank BC000866.1) and human TIMP-2 (GenBank BC052605.1) were synthesized by Mycrosinth (Switzerland). Two different sequences of siRNA were randomly used in the experiments to exclude unspecific RNA interference effects. siRNA against firefly luciferase was used for control transfection. siRNA sequences (5'-3'):
siTIMP-1#1 GAU GUA UAA AGG GUU CCA AdTdT [178]
siTIMP-1#2 GAU GAC CAA GAU GUA UAA AdTdT (designed, Microsynth siRNA Design Tool)
siTIMP-2#1 GGC ACA UUA UGU AAA CAU AdTdT [178]
siTIMP-2#2 GCA GAU AAA GAU GUU CAA AdTdT (designed, Microsynth siRNA Design Tool)
siLuc CGT ACG CGG AAT ACT TCG AdTdT (Microsynth).

pEYFP-TIMP-1 and pECFP-MMP-9 were generous gifts of Dr. M. Roderfeld (Justus Liebig University Giessen). pEGFP (a discontinued product of Clontech) was generously donated by B. Malevich (University of Zurich). TIMP-1 and MMP-9 were subcloned into the EGFP vector to obtain pEGFP-TIMP-1 and pEGFP-MMP-9.

2.14 Microscopy

For wide-field and confocal fluorescent microscopy staining procedure were as follows: cells were cultured on glass cover slips for microscopy, washed with PBS, fixed with 4 % formaldehyde 20 min at RT, permeabilized with 90 % methanol 5 min at RT and blocked in 3 % FCS in PBS for 30min at RT. Incubation with primary antibodies was for 1 h at RT and with secondary fluorofore-conjugated antibodies for 40 min at RT in the dark. Stained cover slips were fixed one more time with 4 % formaldehyde for 10 min at RT and then mounted on glass microscopy slides (Menzel Gläser SuperFrost) with ProLong® Gold Antifade Reagent (Invitrogen). Slides were examined with Leica SP5 confocal microscope or with Zeiss Axiovert 25 microscope equipped with AxioCam MRm (Zeiss) camera.

2.15 Statistical analysis

Statistical analysis was performed using GraphPad Prism 3-5 software (GraphPad Software, Inc.). A comparison between the groups was performed using t-test, the level of significance was set at $P<0.05$.

3 Results

3.1 Cytotoxic effects of patupilone and ionizing radiation, applied alone and in combination

3.1.1 Treatment with patupilone and ionizing radiation has an additive inhibitory effect on cell proliferation

To assess the effect of patupilone and IR on cell proliferation, an MTT-like assay based on the detection of cellular metabolic activity was performed. HT1080 human fibrosarcoma, U251 human glioma and HCT116 human colon carcinoma cells were treated with increasing doses of patupilone and ionizing radiation. All 3 tested cell lines displayed average sensitivity to patupilone in a low nanomolar range, with an IC50 being approximately 0.4 nM for HT1080 and U251 cells and 1.5 nM for HCT116 cells (Fig. 3.1). The radiosensitivity of the tested cell lines differed to a greater extent. HT1080 cells were the most radioresistant. Even a dose of 10 Gy did not inhibit proliferation of these cells by more than 30 %. An IC50 of approximately 5 Gy could be determined for the more radiation sensitive U251 and HCT116 tumor cells.

Treatment with patupilone and IR has an additive effect on tumor cell proliferation as demonstrated in several studies [79, 157]. As such, we tested whether a combined treatment with patupilone and IR also has an additive anti-proliferative effect in our cell systems (Fig. 3.1, bottom). Cells were pre-treated with patupilone for 24 h prior to irradiation. In all three cell lines, an additive anti-proliferative effect was observed with patupilone already at very low concentrations, far below IC_{50}. This was observed in HT1080 cells after treatment with 0.2 nM and 2 or 10 Gy of IR, in U251 cells after treatment with 0.25 nM of patupilone and 2 Gy of IR, and, very prominently, in HCT116 cells after combined treatment with 1 nM patupilone and 2 Gy of IR (Fig. 3.1, bottom).

3.1.2 Patupilone and ionizing radiation have a supra-additive inhibitory effect on clonogenic cell survival

To characterize the long-term effect of patupilone and IR on cell growth, clonogenic cell survival assays were performed with HT1080, U251, HCT116, DAOY, D425 and D341 cells (DAOY, D425 and D341 are human medulloblastoma cell lines). In terms of cell sensitivity to IR and patupilone, results of the clonogenic assays correlated with the results of the cell

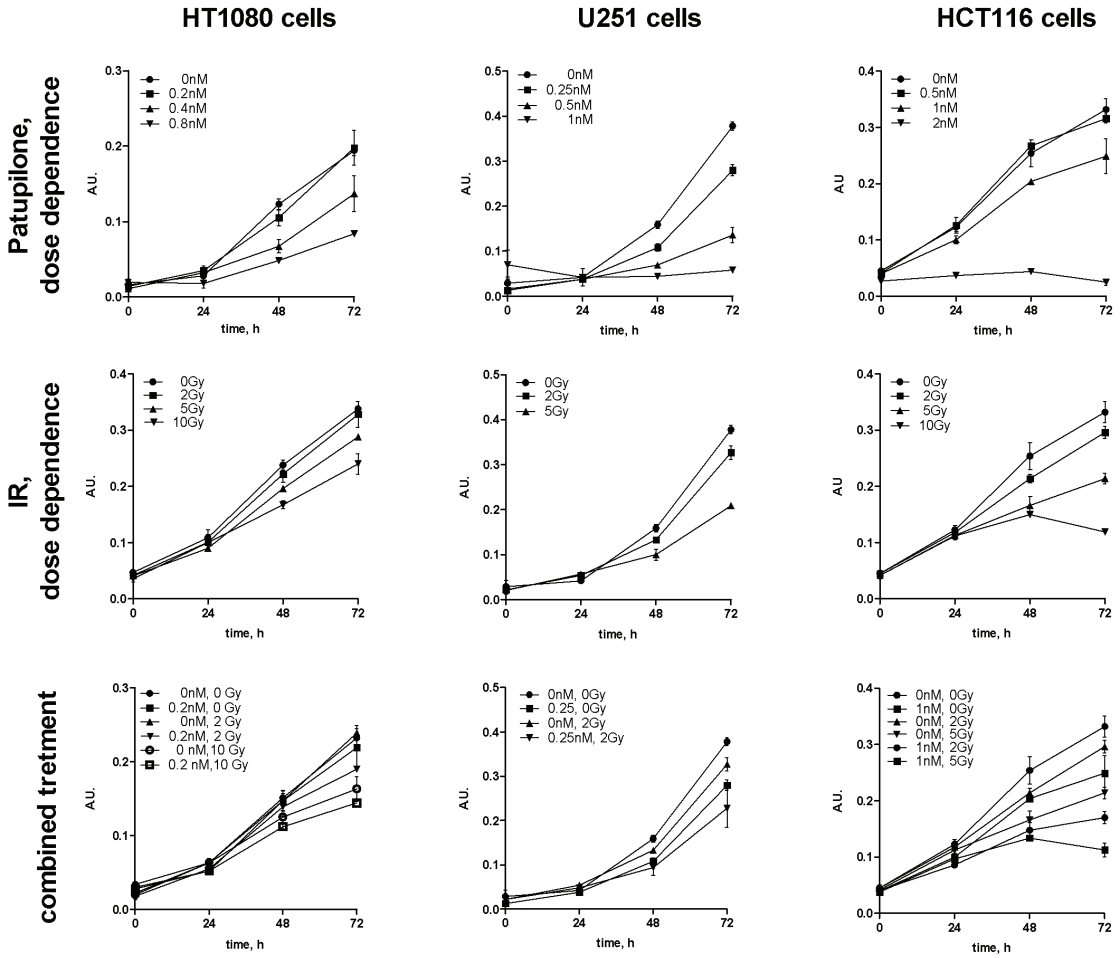

Figure 3.1. Cell proliferation after treatment with the increasing concentrations of patupilone and the increasing doses of IR. In case of the combined treatment the human tumor cells were treated with patupilone 24 h prior to IR. Mean +/- SD, representative assays from at least 3 independent experiments are shown.

proliferation assays described above (Fig. 3.1). HT1080 cells were most radioresistant as irradiation with 2 Gy had no effect on clonogenic survival and the survival fraction after 10 Gy of IR was still 0.3 (Fig. 3.2). In these cells, 0.2 nM patupilone had almost no effect on cell clonogenicity. Likewise, U251 and HCT116 cells were resistant to treatment with 0.05 nM and 0.5 nM of the compound, respectively. DAOY and D425 medulloblastoma cells were highly sensitive to patupilone. Clonogenic survival of these cells was reduced to almost 1 % after treatment with picomolar (60-80 pM) concentrations of patupilone.

The radiosensitizing effect of patupilone (defined as supra-additive effect on cell survival after treatment with the compound and IR) was pronounced when determined on the level of cell clonogenicity (Fig. 3.2). A supra-additive inhibitory effect on clonogenic cell survival was observed in the fiborsarcoma cell line when patupilone was combined with 10 Gy of IR (D (factor of synergy) = 2-9.8 for 0.1-0.8 nM, respectively). In U251 cells and HCT116 cells, a supra-additive effect was observed with 0.2-0.6 nM and 0.5-1 nM patupilone, respectively, applied in combination with 2 and 4 Gy. In these two cells lines, the treatment with the highest

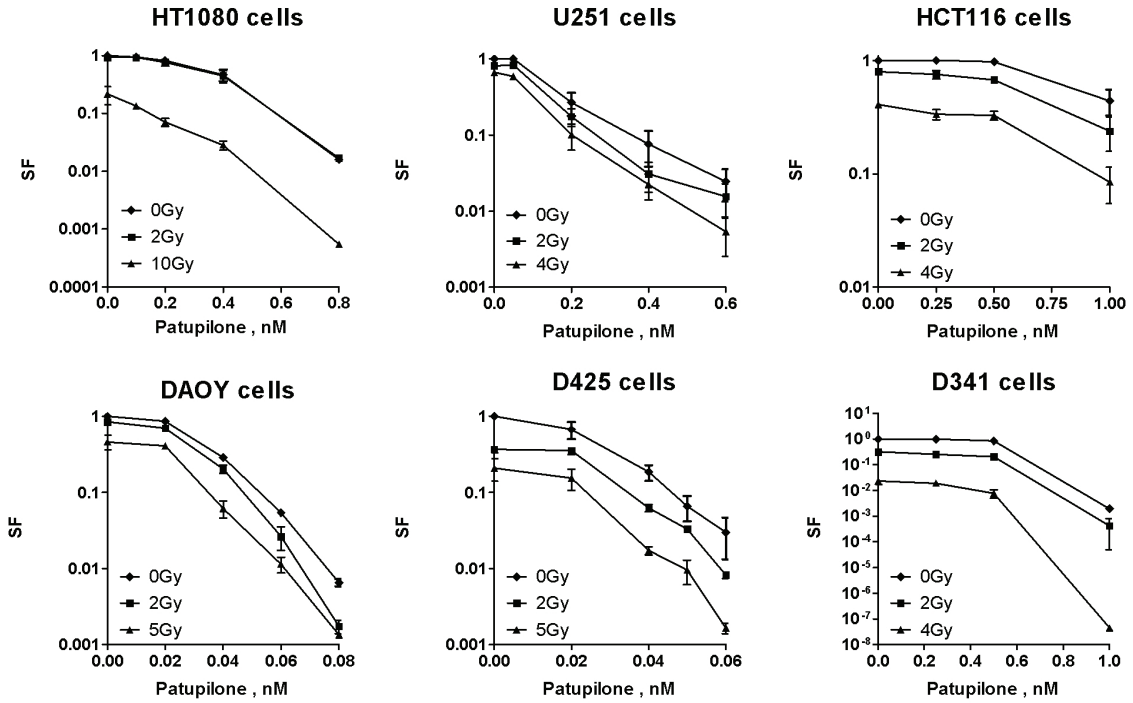

Figure 3.2. Supra-additive anti-clonogenic effect of patupilone administered in combination with IR. The conogenic survival was determined after treatment with the increasing doses of patupilone applied 24 h before irradiation. Mean +/- SE, n=3.

tested concentrations of patupilone applied in combination with 4 Gy IR resulted in D = 1.5 and 3.6 for U251 and HCT116 cells, respectively. Combined treatment reduced clonogenicity in all medulloblastoma cell lines in an at least additive way. Patupilone exhibited a very strong radioenhancing effect (with max D = 1000) in D341 medulloblastoma cells at concentrations below 1 nM when applied together with 4 Gy IR.

3.1.3 Low concentrations of microtubule inhibitors did not affect morphology of microtubule cytoskeleton

The mechanisms of cytotoxicity of MTSAs are dose-dependent. At high doses, MTSAs prevent depolymerisation of MT. Morphologically, it manifests by occurrence of MT-bundles in the cytoplasm and leads to abrogation of mitosis due to prevention of spindle formation. After treatment with lower doses of MTSAs, cytoskeletal structures retain normal morphology; however, MT dynamics (the rate of growth and shortening) can be inhibited and cells may lose clonogenicity as a result of aberrant mitotic divisions [61, 68-69]. Therefore, microscopic evaluation of MT structures in the cell provides the first clues for the mechanism of action of MTSAs.

The morphology of the microtubule cytoskeleton of HT1080 cells was assessed by fluorescent microscopy. The HT1080 cells were chosen for the experiment as they are a well-established *in vitro* system for studies of MMP function and cell invasion and most

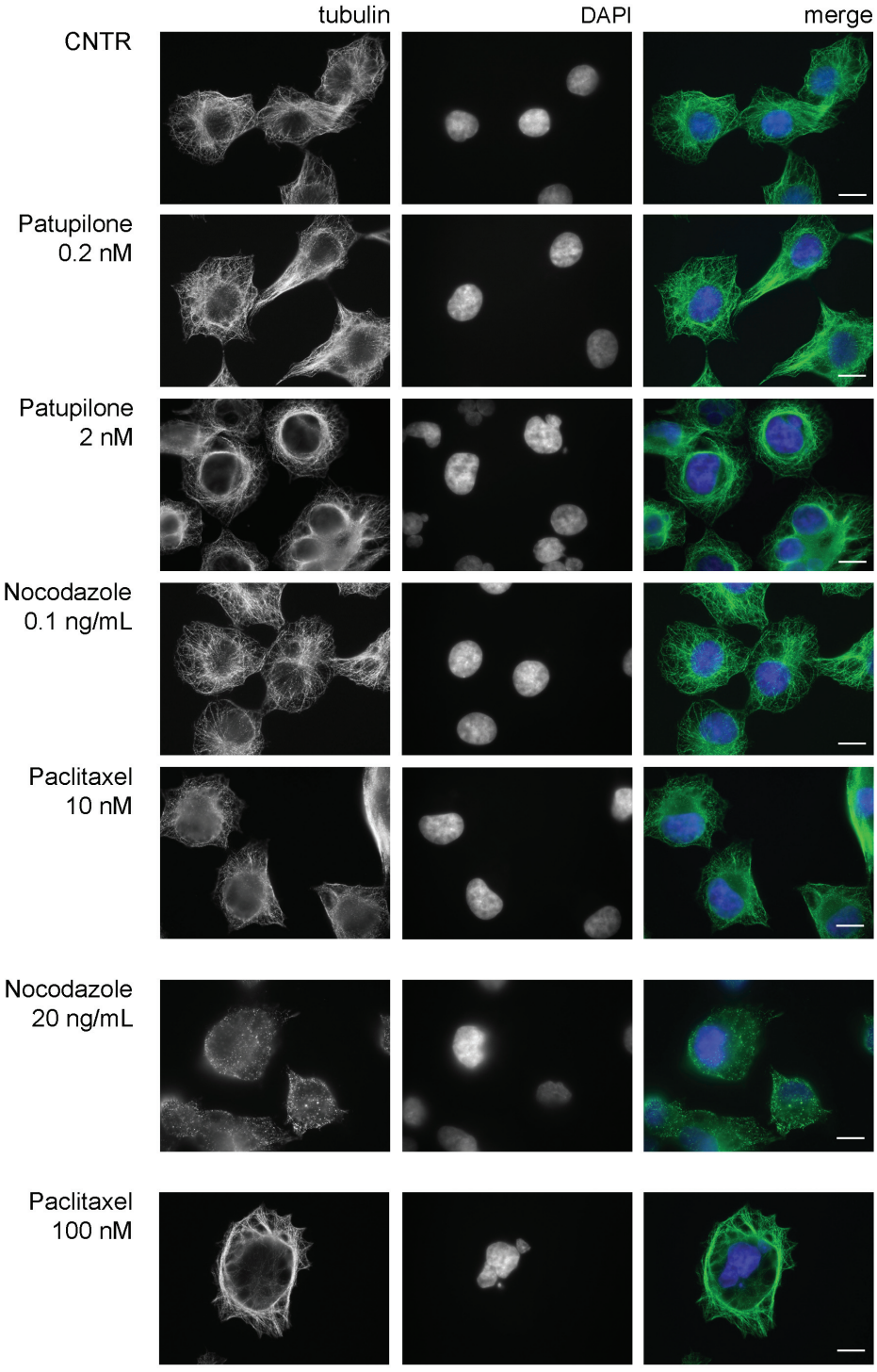

Figure 3.3. The MT morphology in HT1080 cells after treatment with MT-interfering agents. 24 h and 48 h after treatment with 0.2 and 2 nM patupilone, respectively; 14 h after nocodazole treatment; 24 h after taxol treatment. The MT bundling occurs with 100 nM paclitaxel. The MT disruption occurs with 20 ng/mL nocodazole. Green – β-tubulin, blue – DAPI, scale bar 10 µm.

of following work was performed with this cell line. The cells were treated with different concentrations of patupilone and other MT-inhibitors, such as paclitaxel and nocodazole (MT destabilizer). A classical microtubule bundle formation was observed after 24 h treatment

with 100 nM paclitaxel and, to a lower extent, after incubation of cells with 2 nM patupilone (Fig. 3.3). Treatment with lower doses of patupilone and paclitaxel did not lead to changes in MT morphology. No changes were observed after treatment with 1 ng/mL (non-toxic concentration) of nocodazole, in comparison to the response to a higher cytotoxic concentration (20 ng/ml), which led to a complete disruption of the MT in the cells.

Our laboratory previously demonstrated that patupilone is a potent radiosensitizer in vitro and in vivo in different human cancer cell systems [79, 157]. The results presented herein further confirmed the ability of patupilone to enhance IR-induced cytotoxicity (Fig. 3.2). Of additional interest, is the fact that patupilone has a radiosensitizing capacity at very low doses that are not cyctotoxic themselves and do not alter the MT cytoskeleton structure (0.2 nM patupilone in HT1080 cells, Fig. 3.3).

3.2 Patupilone counteracts ionizing radiation-dependent enhancement of MMP function

3.2.1 Patupilone inhibits ionizing radiation-induced MMP activity in conditioned cell culture media

The enzymatic activity of MMPs is essential for their role in a vast majority of pro-tumorigenic processes. The repertoire of MMP substrates extends beyond growth factors, regulatory proteins and protein components of the extracellular matrix (ECM). By cleaving their substrates, MMPs can regulate nearly all stages of tumor development, including, for example, cell growth and apoptosis, angiogenesis and metastasis (see Chapter 1.4.5). Moreover, there is strong evidence that IR can promote MMP expression (see Chapter 1.2.2) and, therefore, tumor aggressiveness. As radiation therapy is one of the most prevalent cancer treatment modalities, it is important to take into consideration its potentially unfavourable effects, such as the induction of MMPs. Combination therapies aimed at counteracting these unfavourable effects are therefore of a substantial interest. MTSAs are potential candidates for such a combination, as they can inhibit MMP secretion [48, 174].

Herein, we probed the influence of patupilone and IR treatment on MMP activity. As MMPs are secreted proteins and enzymaticaly active in the extracellular space, we used a FRET-peptide based assay to determine MMP activity in conditioned cell culture media (CM). The assay is not specific for individual MMP activity but allows to measure pooled MMP activity without pre-processing the sample. The determination of the “native” activity of secreted MMPs is important as it can be translated directly in most of MMP biological functions [139].

MMP activity was assessed in CM of HT1080, U251 and HCT116 cells after treatment with IR and patupilone, alone and in combination (Fig. 3.4). IR increased MMP activity in

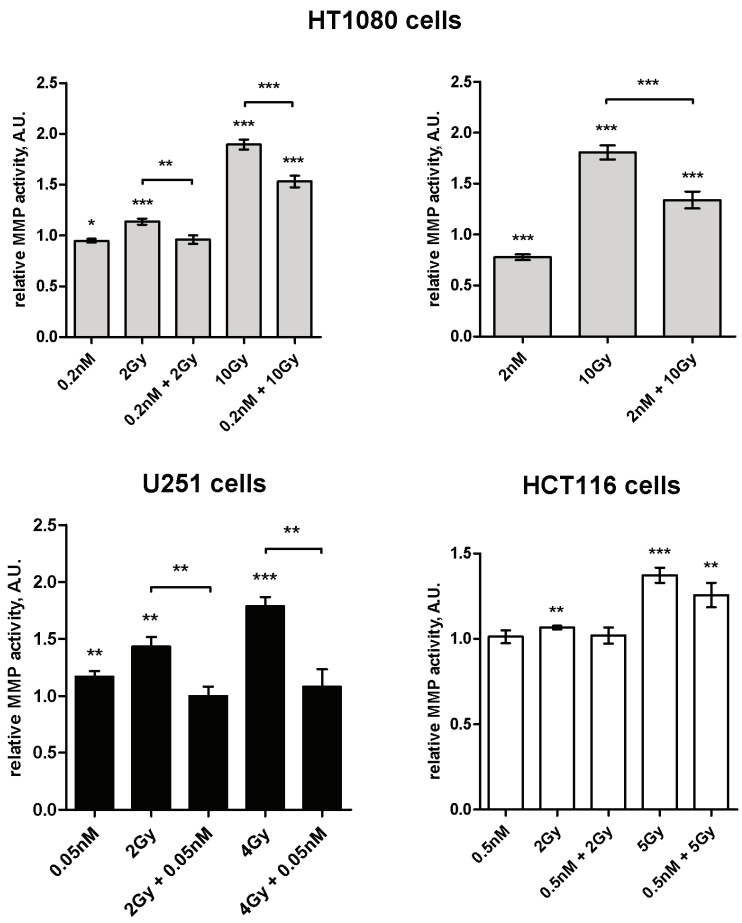

Figure 3.4. Patupilone at the non-cytotoxic concentration inhibits the MMP activity induced by IR in the cell culture media supernatant. The cells were treated with the indicated concentrations of patupilone 24 h prior to IR or 2 nM of patupilone 1 h after IR, the MMP activity was measured 24 h after IR treatment. The results are plotted as mean +/- SE. N=13 for HT1080 cells, n=6 for U251 cells, n≥4 for HCT116 cells, *P<0.05, **P<0.01, ***P<0.001.

CM, determined 24 h after irradiation, in a dose dependent manner in all three cell systems tested. The highest dose of IR, which was used in our experimental setup, decreased the amount of cells by 30-40 %; as such, the measured MMP activity was corrected to the cell number. We observed a 1.9 and 1.8-fold increase of MMP activity in CM of HT1080 and U251 cells after irradiation with 10 Gy and 4 Gy, respectively (P>0.001). A 1.5-fold increase of MMP-activity was detected in CM of HCT116 cells after irradiation with 5 Gy (P>0.001). An increase of MMP activity in CM of all three cell lines was also observed after treatment with cytotoxic doses of patupilone (higher than 2 nM, data not shown).

Treatment with patupilone at low doses (doses that did not lead to significant decrease of cell proliferation and clonogenic survival: 0.2 nM for HT1080 cells, 0.05 nM for U251 cells and 0.5 nM for HCT116 cells) had various effects on MMP activity. In HT1080 cells the MMP activity was reduced by 5 % (P=0.011). In U251 cells MMP activity was increased by 15 % (P=0.004). No effect on MMP activity after treatment with low dose patupilone was observed in HCT116 cells (Fig. 3.4). Thus the influence of patupilone treatment on basal level of MMP activity was minimal. A more pronounced effect of patupilone was observed on the IR-induced MMP activity levels. MMP activity in U251 cells was elevated 1.4- and 1.8-fold after irradiation with 2 and 4 Gy respectively. Pre-treatment with 0.05 nM of patupilone completely abrogated

IR-induced MMP activity in these cells (P=0.0037, Fig. 3.4). Pre-treatment of HT1080 cells with 0.2 nM of patupilone diminished irradiation-induced elevation of the MMP activity to 1.5-fold, compared to an almost 2-fold increase after IR treatment alone (P<0.0001). A similar tendency was determined in HCT116 cells, even though MMP activity was not significantly decreased after combined treatment with 5 Gy and 0.5 nM patupilone, compared to irradiation treatment alone.

Patupilone accumulates in cells during long-term incubation [60]. This pharmacodynamic feature can lead to the following phenomena: a longer incubation with low dose of the compound leads to the same biological outcome as a shorter incubation with a higher dose. To probe our results observed in HT1080 cells after treatment with 0.2 nM patupilone for 24 h prior to IR, we performed a similar experiment with 10-fold higher concentrations of patupilone (2 nM). The high concentration of the compound was applied immediately after irradiation (no pre-incubation) and MMP activity was assessed in 24 h. During such a short experimental time, incubation with 2 nM patupilone did not lead to cytotoxicity or a decrease in the cell number. In this experimental setup, we again observed patupilone counteracting IR-induced MMP activity elevation in CM (Fig. 3.4). The MMP activity was elevated only 1.5-fold after combined treatment with 10Gy and 2 nM, compared to almost 2-fold increase after IR treatment alone (P=0.0008).

3.2.2 Patupilone and ionizing radiation do not influence membrane-bound MMP activity

The MMPs can be anchored to the outer cell membrane. First, there are membrane type MMPs (MT-MMPs) which are trans-membrane proteins. Second, secreted MMPs may be attached to the cell membrane through various receptors on the cell surface. The MMPs on the outer cell surface are relevant for pro-MMP activation and for the cell migration as they

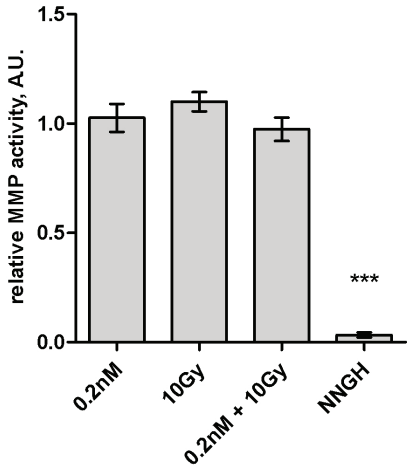

Figure 3.5. Patupilone and IR have no effect on the membrane-bound MMP activity. HT1080 cells were treated with patupilone and IR as indicated in Fig. 3.4 or with 10 μM of NNGH. The results are plotted as mean +/- SE, n=5, ***P<0.001.

can degrade ECM (rev in [96]). We therefore assessed membrane-bound MMP activity after treatment with 0.2 nM patupilone and 10 Gy by exposing HT1080 cells to the FRET MMP substrate that was well tolerated by the cells. The rate of substrate cleavage activity measured in this experimental setup was 20-fold higher than in CM. The measured signal was attributed to MMP activity as it could be completely abolished by the specific MMP inhibitor NNGH (N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid) (Fig. 3.5).

No change in cell-associated MMP activity could be determined after treatment with patupilone and IR, indicating that only protease activity, derived from secreted MMPs, is affected by the investigated treatment modalities.

3.2.3 Effect of microtubule inhibitors on ionizing radiation-induced MMP activity in conditioned cell culture media

We hypothesized that the counteracting effect of patupilone on IR-induced MMP activity may be due to its ability to inhibit the MT function. If MT-inhibitory properties of the compound play an important role, then other MT inhibitors should demonstrate a similar counteracting effect on IR-induced MMP activity. Therefore, we tested the ability of the clinically relevant MT stabilizer paclitaxel and two MT destabilizers, vincristine and nocodazole, to inhibit IR-induced MMP activity in CM of HT1080 cells.

Paclitaxel, at a concentration of 10 nM, strongly reduced the basal MMP activity level (by 40 %) and decreased IR-induced MMP activity to basal level (Fig. 3.6, left). Increasing concentrations of vincristine were tested but we could not determine any effect of low concentrations of the compound on MMP activity in CM of HT1080 cells. Vincristin

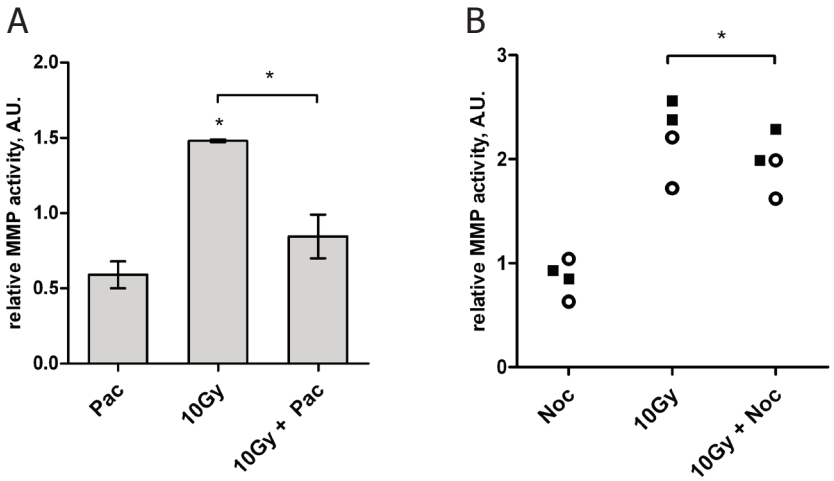

Figure 3.6. Microtubule inhibitors diminish the IR-induced increase of the MMP activity in the cell culture media supernatant. HT1080 cells were treated with **(A)** 10 nM paclitaxel (Pac) after IR, the results are plotted as mean +/- SE, n=2; or **(B)** 1 ng/mL nocodazol after IR for 4 (circles) or 14 (squares) hours. The MMP activity was measured 24 h after IR. *P<0.05.

at higher concentrations upregulated MMP activity (data not shown). On the other hand, enhancement of MMP activity by IR was diminished when cells were treated with another MT destabilizer nocodazole. This effect was small, but significant (P=0.026, Fig. 3.6, right). Thus MT stabilizers might have a more pronounced inhibitory effect on MMP activity in CM than MT destabilizers. Importantly, even very high concentrations of patupilone (100 nM) did not inhibit the activity of purified recombinant MMP-2 and -3 (data not shown). Therefore, the observed inhibition of MMP activity in CM after patupilone treatment has to be attributed to cellular processes.

3.2.4 Differential effects of patupilone and ionizing radiation on cell motility and invasion

We demonstrated that patupilone and IR variously altered MMP activity levels in CM. Furthermore, patupilone, at a low non-cytotoxic concentration, can counteract IR-induced MMP activity. The question remains whether the observed effect is of a biological relevance. To test this, we assessed the cell invasion in a transwell chamber assay after corresponding treatments. The cell invasion does not reflect all the complexity of MMP functions because the MMPs play a role in the regulation of many other cellular processes, including apoptosis, angiogenesis and so forth [179]. However, the transmigration assay is highly specific for MMPs as it requires extracellular matrix (ECM) degradation [180]. In addition to the ECM proteolysis, a certain level of cell motility is required for successful cell invasion.

Transwell chamber assays were performed with HT1080, U251 and HCT116 cells to assess their migration capacity after treatment with patupilone and IR. To mimic a process of tumor cell invasion through the basal membrane, Matrigel™-coated transwell inserts were used. Cell motility was evaluated in non-coated inserts. The three tested cell lines were different in their basal rate of migration (see Table 3).

Table 3. Migration and invasion capacity of human tumor cells.

Cell line	Cells migrated through the pores of the transwell insert in 24h	Cells invaded through Matrigel™ in 24h
HT1080	100%	10-20%
U251	20%	1-5%
HCT116	~1%	<0.1%

The migration capacity of the cells was tested after treatment with increasing concentrations of patupilone and dosed of IR. Patupilone at non-antiproliferative concentrations (see above, Fig. 3.1, 3.2) (< 0.2 nM; 0.025 nM and 0.1 nM) and low doses of IR (2 Gy; 1 Gy and 2 Gy) did not affect the motility of the HT1080, U251 and HCT116 cells, respectively (Fig. 3.7).

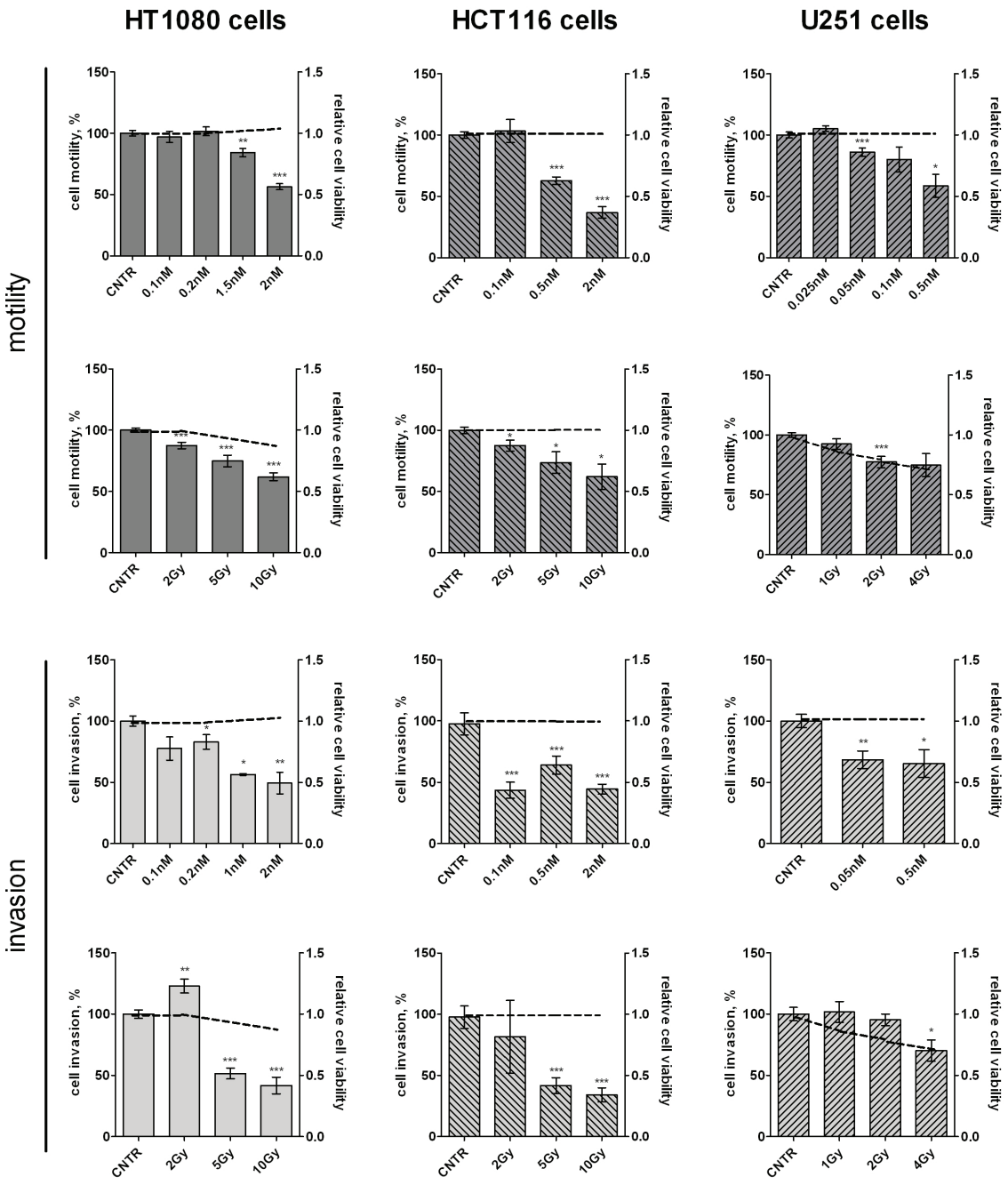

Figure 3.7. Patupilone and IR have various dose-dependent effects on the cell motility and invasion. The cell invasion and motility was assessed in the transwell assay 24 h after the indicated treatments. Bars and the left Y-axis - the percentage of the motile/invading cells relative to control, mean +/- SE, N>3, *P<0.05, **P<0.01, ***P<0.001. The dashed line and the right Y-axis – the amount of viable cells determined under the same conditions.

Treatment with higher concentrations of patupilone, as well as higher doses of IR, inhibited cell motility by up to 50 % in all three cell systems in a dose dependent way. High doses of patupilone and IR, which inhibited cell motility, also resulted in decreased rates of invasion. The low dose IR, on the contrary, increased cell invasion in HT1080 cells by approximately 30 %, even though cell motility was slightly decreased. In U251 cells, invasion after irradiation

with 2 Gy was similar to control but cell motility at this dose was reduced by almost 30 %. Interestingly, the invasion of HT1080 and HCT116 cells was already inhibited by patupilone at a concentration that did not have any effect on cell motility or viability, namely 0.1 nM. This effect cannot be due to reduced cell motility and therefore might be attributed to a decreased ability of the cells to degrade extracellular matrix.

The cell migration capacity under different conditions was determined within 24 hours. In such a short experimental time, no quantitative changes with regard to cell viability occurred after treatment with patupilone at tested concentrations (≤ 2 nM for HT1080 and HCT116 cells and ≤ 0.5 nM for U251 cells). After treatment with IR (dose of 2 Gy and more), the number of HT1080 and U251 cells decreased in a dose dependent way, most probably due to IR-induced cell cycle arrest, since the amount of dead cells in the trypan blue viability assay was negligible. Thus, the observed decrease in cell motility after irradiation, as determined in the transwell chamber assay, could result from a decreased amount of cells.

3.2.5 Patupilone inhibits ionizing radiation-induced tumor cell "invasive capacity"

As described above, low dose IR inhibited cell motility. However, cell invasion was not decreased in U251 cells and even promoted in HT1080 cells. To further analyse our results, we determined the "invasive capacity" of the cells, which was defined as follows: (amount of invading cells) / (amount of motile cells). This approach allowed us to focus on motility-independent rates of cell invasion given by the ability of the cells to degrade the Matrigel™. This analysis revealed that treatment with low dose IR (2 Gy), resulted in an increase of invasive capacity by 50 and 30 % for HT1080 and U251 cells, respectively (Fig. 3.8). Pre-treatment of cells with patupilone completely abolished IR-induced invasion, and cells that had been treated with a combination of patupilone and IR exhibited the same invasive capacity as untreated control cells. The treatment of cells with patupilone alone led to a slight

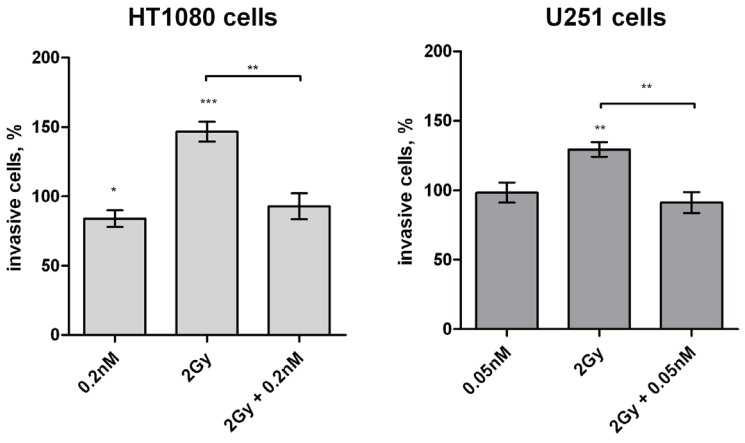

Figure 3.8. Patupilone counteracts the IR-dependent induction of cell invasion. The cells were plated in the transwell inserts in the presence of the indicated concentrations of patupilone 4 h prior to irradiation. The rate of invasion was evaluated 24 h after plating. The results are plotted as percentage of the invading cells relative to control, mean +/- SE, n≥3, *P<0.05, **P<0.01, ***P<0.001.

decrease of the basal cell invasion in HT1080 cells.

Overall the effects of IR and patupilone on cell invasion were well correlated with the effects of the corresponding treatments on MMP activity in CM (Fig. 3.4). Both on the level of MMP activity and cell invasion, the following results were observed: a decrease of MMP activity and cell invasive capacity after treatment with patupilone alone (in case of HT1080 cells), an increase after irradiation and the counter effect of patupilone on IR-induced MMP activity and cell invasion.

3.2.6 MMP enzymatic activity is required for cell invasion

As outlined, we observed a correlation between the changes in the invasive capacity of tumor cells and MMP activity in their CM, in response to the different treatment modalities (IR and patupilone, alone and in combination). In order to mechanistically link these processes we performed transwell invasion experiments in the presence of MMP inhibitors: recombinant TIMP-1 and -2 (rTIMP-1, rTIMP-2, natural non-cell permeable inhibitors of MMPs) and a small molecule cell-permeable MMP-specific inhibitor NNGH.

First, the efficacy of the inhibitors was tested in the MMP assay with recombinant purified MMP-2 (rMMP-2), MMP-9 (rMMP-9) and CM from HT1080 cells. NNGH potently inhibited the MMP activity in all tested systems (Fig. 3.9). The activity of rMMPs was strongly inhibited by rTIMPs; however, rTIMPs did not affect the MMP activity in CM, even at concentrations 10-times higher than required for the inhibition of the activity of recombinant MMP-2 and -9. As MMPs may be secreted in a complex with endogenous TIMPs [181], we speculated that this might prevent binding of exogenous rTIMPs and subsequent MMP activity inhibition. To prove this hypothesis, we down-regulated endogenous TIMP-1 or TIMP-2 in HT1080 cells with siRNA (Fig. 3.18) and determined MMP activity in their CM, in both the presence and absence of rTIMPs. However, the inhibition of MMP activity by rTIMPs in this CM could

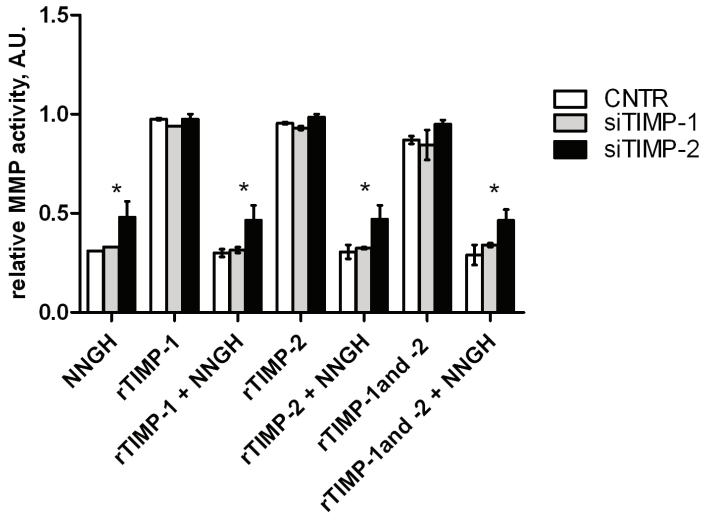

Figure 3.9. The MMP inhibitor NNGH but not recombinant TIMP-1 and TIMP-2 proteins inhibit the MMP activity in CM. NNGH, rTIMP-1 and rTIMP-2 were added to CM at concentrations 10 μM, 0.16 μM and 0.4 μM, respectively. The cell were transfected with siTIMP-1 or siTIMP-2. siLuc was used as an irrelevant control siRNA. Data is shown as mean +/- SE, n=2, *P<0.05.

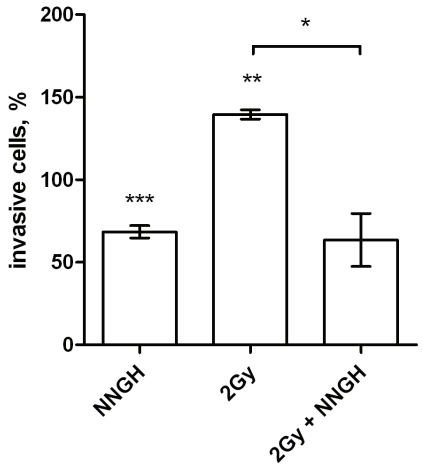

Figure 3.10. The MMP inhibitor NNGH inhibits the cell invasion. The cells were plated with 10 μM NNGH 4 h prior to irradiation. The rate of invasion was evaluated 24 h after plating. The results are plotted as percentage of the invading cells relative to control, mean +/- SE, n≥3, *P<0.05, **P<0.01, ***P<0.001.

not be observed. Moreover, rTIMP-2 partly lost its ability to inhibit activity of rMMPs after pre-incubation with CM of HT1080 cells (data not shown). This indicated that there might be a TIMP-scavenging/neutralising activity in cell culture media supernatant of HT1080 cells.

The addition of rTIMPs to HT1080 cells in the transwell assay did not affect cell invasion or migration significantly (data not shown), as it did not inhibit MMP activity in CM (Fig. 3.9). However, the treatment of cells with the small molecular MMP inhibitor NNGH, led to a reduction of cell invasion to 50-60 % of the control level (Fig. 3.10). This reduction of cell invasion occurred both in cells irradiated with 2 Gy and non-irradiated cells. This finding indicates that in our experimental system, cell invasion required MMP activity.

The combined treatment modality with patupilone and IR is more potent in vivo than in cellular model systems. Most probably this is due to the additional treatment effects on the level of the tumor microenvironment, which may be mediated by tumor cells [79, 157]. Herein, we investigated an effect of the indicated treatment regimens on one of the important components of the tumor microenvironment, namely MMPs. We identified that the activity of secreted MMPs was up-regulated after treatment with IR and that pre-treatment with patupilone could diminish this increase (Fig. 3.4). MMP activity is required for tumor cell invasion as well as for other cellular processes. In this study, tumor cell invasive capacity was indeed increased after IR and, in line with the events on the level of MMP activity, patupilone could counteract this effect of IR (Fig. 3.8).

3.3 The mechanisms of inhibition of ionizing radiation-induced MMP activity by patupilone

3.3.1 Patupilone does not prevent ionizing radiation-induced cell cycle arrest

Both ionizing radiation and microtubule stabilizing agents may alter the cell cycle distribution of the treated cells in a time-, dose- and cell line-dependent way. Ionizing radiation induces cell cycle arrest by activation of the G2-checkpoint in tumor cells with subsequent accumulation of cells in the G2 phase of the cell cycle [20]. Patupilone has various, dose-dependent effects on cell cycle distribution. A strong accumulation of cells in G2/M-phase occurs after treatment with high cytotoxic doses [153]. After treatment with lower doses both S phase accumulation [79] and G1 arrest [182] were observed. We hypothesized that the counter effect of patupilone on IR-induced MMP activity may be due to the influence on the cell cycle distribution, for example, the compound might prevent G2/M accumulation after irradiation.

We assessed the cell cycle distribution of HT1080 and HCT116 cells after treatment with patupilone and IR by flow cytometry. No change in cell cycle distribution was observed in HT1080 cells after treatment with either 2 Gy or 0.2 nM of patupilone (Fig. 3.11). Irradiation with 10 Gy induced accumulation of cells in G2/M phase, which occurred within 12 h after treatment. Patupilone, even after treatment with a very high cytotoxic dose, namely 2 nM, only minimally affected cell cycle distribution, inducing only minor accumulation in S phase: 39 % (treated) vs. 29 % (control) (Fig, 3.11A). After combined treatment with 10 Gy of IR and 0.2 nM patupilone cell cycle distribution was similar to the one after treatment with radiation alone (Fig. 3.11B, left). In HCT116 cells, similar results were observed. Treatment with 0.5 nM patupilone did not affect cell cycle distribution (Fig. 3.11B, right). Only after treatment with very high concentrations of patupilone, namely > 20 nM (2 nM is patupilone IC_{100} in HCT116 cells), could the classical G2/M phase arrest be detected. Treatment with 2 nM patupilone led to accumulation of cells in S-phase. G2/M accumulation was observed after 5Gy IR alone and in combination with 0.5 nM patupilone (10-13 % of cells in G2/M phase after IR vs. 6 % of non-irradiated cells).

Based on these observations we can conclude that the counteracting effect of patupilone on IR-induced MMP activity and cell invasion is not due to cell cycle-related mechanisms, as patupilone at the corresponding concentrations did not influence cell cycle distribution of non-irradiated HT1080 and HCT116 cells and did not prevent G2/M accumulation after IR.

3.3.2 Cell cycle-related changes of MMP activity in conditioned cell culture media

IR induced MMP activity in the CM and cell cycle arrest with an accumulation of cells in G2/M phase. We questioned if cell cycle arrest was a sufficient factor that could induce the MMP activity levels in response to IR. To approach this question, HT1080 cells were treated

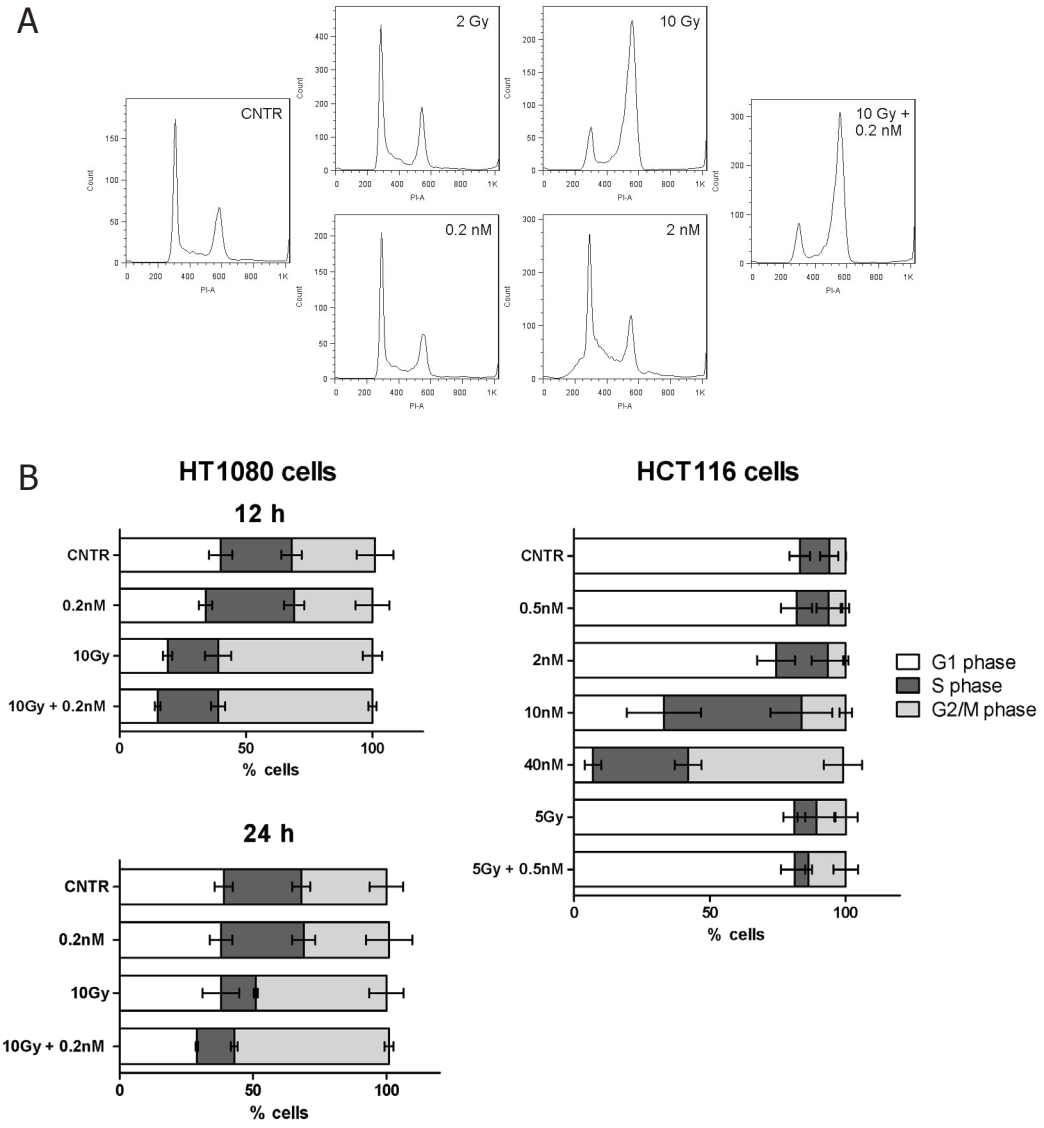

Figure 3.11. Cell cycle distribution after the treatment with patupilone and IR. The cell cycle distribution was assessed after the indicated treatments. Patupilone was administered 24 h prior to IR. The cell cycle distribution was assessed in HT1080 12 and 24 h after IR; in HCT116 cells 24 h after IR.
Hystograms – the representative cell cycle distribution of HT1080 cells (A); bar diagrams – mean +/- SE, n=3 (B).

with hydroxiurea (HU) and aphidicolin (Aph) – chemical compounds inducing S phase accumulation [43]. The accumulation of cells in the S phase of the cell cycle was confirmed by flow cytometry (data not shown) and MMP activity in CM of cells in S phase was determined.

Treatment of cells with both compounds resulted in the induction of MMP activity in CM. The increase after incubation with 1mM HU was 3.7-fold, and 15-fold after 4 μg/mL Aph (Fig. 3.12). Measurements of MMP mRNA levels showed that MMPs were induced on the transcriptional level (data not shown). Thus, we showed that chemically-induced cell

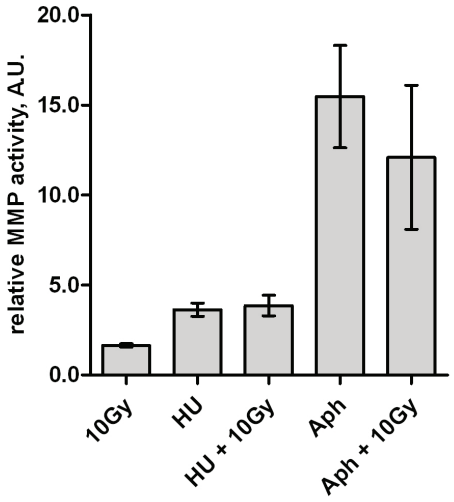

Figure 3.12. The cell cycle arrest induces MMP activity in the cell culture media supernatant. The cells were treated with 1 mM hydroxyurea (HU) or 4 µg/mL aphidicolin (Aph) for 36 h and irradiated 18 h before the MMP activity analysis. Mean +/- SE, n≥2.

cycle arrest in S phase can induce MMP activity in CM, as well as cell cycle arrest in G2/M phase after IR. To test whether the induction of MMP activity in CM after treatment with IR or HU/Aph occurs via similar mechanisms, we irradiated the cells which were arrested in the S phase and determined MMP activity in their CM. As shown in Fig. 3.12, no additive increase of MMP activity was observed after combined treatment with IR and hydroxyurea or aphidicolin. The result suggests that the induction of the MMP activity occurred via related mechanisms, both after irradiation and after S phase arrest.

The results demonstrated that on the one hand the induction of MMP activity after IR is not due to G2/M phase accumulation but rather due to cell cycle arrest, as S phase arrest with hydroxyurea and aphidicolin also induced MM activity. On the other hand, these findings indicated once more that MMP activity induction might be a part of a cellular stress response, which can be induced by different means, e.g. IR, cell cycle arrest or treatment with cytotoxic compounds (see Chapter 3.2.3).

3.3.3 Patupilone does not interfere with MMP expression on transcriptional level

Previous reports claim that ionizing radiation induces MMPs on the level of transcription [100, 183]. As such, we investigated if patupilone can decrease IR-induced MMPs mRNA levels. qRT-PCR was performed with mRNA samples obtained form HT1080 cells treated with 0.2 nM patupilone and IR (2 and 10 Gy), alone and in combination. Transcription of MMP-2, -9 and -14 was induced by approximately 50% 24 h after irradiation with 10Gy ($P>0.05$, Fig. 3.13). Patupilone altered neither the basal level of MMP transcription nor the level of IR-enhanced transcription. We also assessed the mRNA levels of MMP-1 and MMP-3 but did not observe any significant changes after any applied treatments (data not shown). In HCT116 cells, only mRNA levels of MMP-2 and MMP-9 were elevated by 1.8- and 1.5-fold,

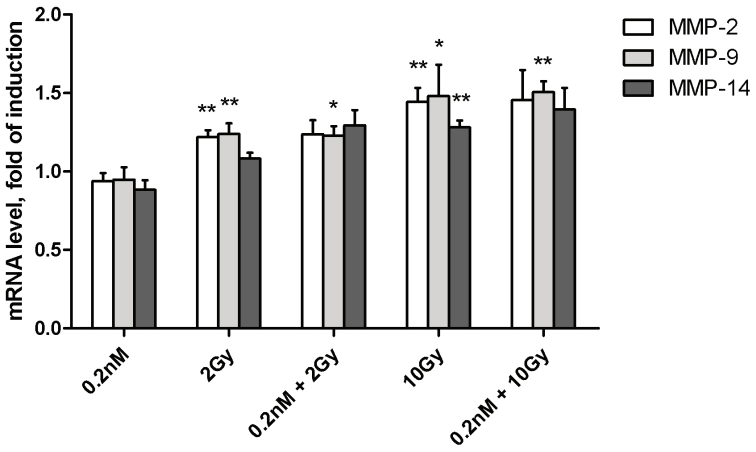

Figure 3.13. Patupilone does not alter MMP mRNA levels. The MMP mRNA levels in HT1080 cells were determined using qRT-PCR 24 h after treatment with IR. Patupilone was applied on cells 24 h prior to IR. Data is shown as mean +/- SE, n≥4, *P<0.05, **P<0.01.

respectively, after irradiation with 5Gy. Patupilone did not effect basal or IR-induced MMP transcription (data not shown).

To confirm that patupilone does not alter induced MMPs transcription, cells were treated with an established transcriptional inducer of MMPs, phorbol-12-myristate-13-acetate (PMA) in combination with patupilone. PMA activates PKC and the activation of its downstream pathways leads to increased transcription of MMP-1, -9, and -14 and TIMP-1 [125-126, 184]. In our experiment, 40 µg/mL PMA upregulated MMP-9 and MMP-1 transcription, 13-fold and 6-fold, respectively. However, we could not detect up-regulation of MMP-14 (Fig. 3.14A). Pre-treatment with patupilone failed to inhibit PMA-induced transcription. Never-the-less, we again observed an inhibitory effect of patupilone on the PMA-induced MMP activity in CM of HT1080 cells (Fig. 3.14B). The proteolytic activity of MMPs doubled after incubation with PMA but was only induced by 1.6-fold when cells were pre-treated with 0.2 nM of patupilone (P=0.0001). Interestingly, the magnitude of PMA-induced MMP activity inhibition

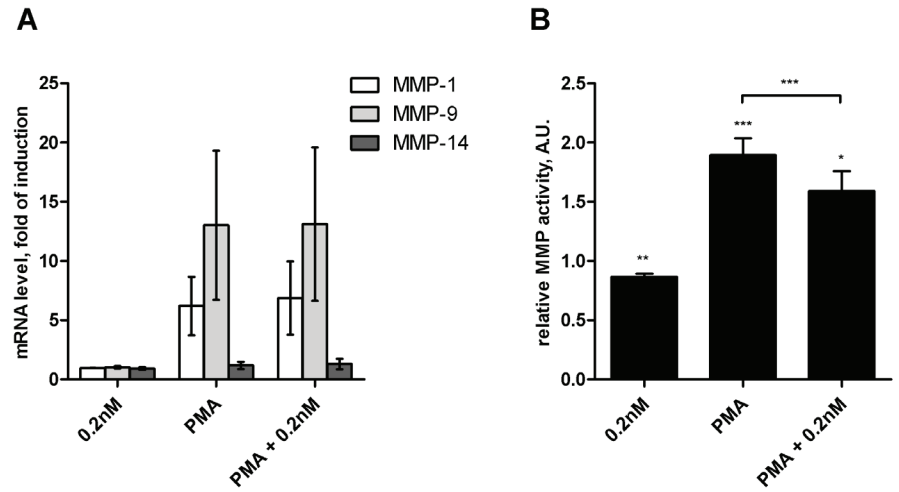

Figure 3.14. Patupilone does not alter the PMA-induced MMP mRNA levels. HT1080 cells were treated with patupilone 24 h before application of 40 µg/mL PMA. 20 hours thereafter, mRNA for qRT-PCR (A) and CM for determination of the MMP activity (B) were isolated. Data is shown as mean +/- SE, n>4, *P<0.05, **P<0.01.

by patupilone was similar to the inhibition observed for IR-induced activity (Fig. 3.4).

3.3.4 Patupilone has no effect on MMP intracellular and extracellular protein levels

No effect of patupilone treatment on the level of MMP transcription was observed. MMP activity, however, is regulated on multiple levels, including proteolytic activation and secretion (see Chapter 1.4.1). Furthermore, MTSAs were shown to inhibit MMP activity in the extracellular space by interfering with the MMP secretion process [48]. To probe whether the treatment with IR and patupilone influence expression or secretion of MMP proteins, we probed intracellular and extracellular protein levels of MMP-1, -2, -3, -9 and -14. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the most relevant MMPs for the first step of cell invasion – breaking the basal membrane. MMP-14 (MT1-MMP) is involved in proMMP-2 activation [108]. MMP-1 (colagenase I) and MMP-3 (stromelysin I) are abundant in the CM of HT1080 cells as we demonstrated by MMP antibody array (Fig. 3.15).

To determine the protein levels of the above-mentioned MMPs after treatment with patupilone and/or IR, we performed gelatine zymography with CM samples and Western blotting with CM and whole cell lysates of HT1080 cells. Gelatine zymography is a very sensitive SDS-PAGE-based technique which allows one to semi-quantitatively determine protein amount of zymogens and active forms of MMP-2 and MMP-9 by measuring their gelatinolytic activity [185]. Due to technical limitations (lack of antibody specificity and sensitivity), we could not detect MMP-1 and MMP-2 by western blotting; MMP-3 was

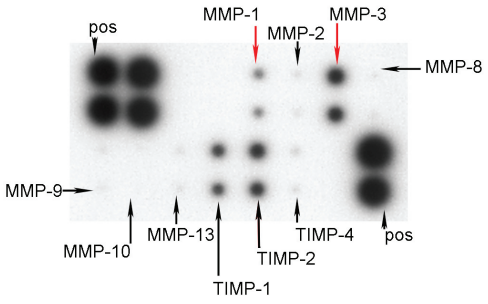

Figure 3.15. MMP and TIMP proteins in CM of HT1080 cells. HT1080 cells were cultured for 24 h in serum free media. The CM was collected as described in Materials and Methods and MMP antibody array was performed. pos – positive control.

detectable only in CM. As irradiation with 2 Gy only minimally affected MMP activity (Fig. 3.4) and mRNA level (Fig. 3.13) in HT1080 cells, we chose a radiation dose of 10 Gy for further studies.

MMP-9 zymogen band could be detected by Western blotting in cell lysate at approximately 100 kDa (predicted size 95-98 kDa) (Fig. 3.16). The MMP-9 band was detected in the CM of HT1080 cells on the level of ~80-90 kDa, which corresponds to the size of the active form of MMP-9 (predicted size 82 kDa). MMP-2 and MMP-3 were also present in CM as active forms; the MMP-2 band was detected at approximately 60-65 kDa (predicted size 62 kDa),

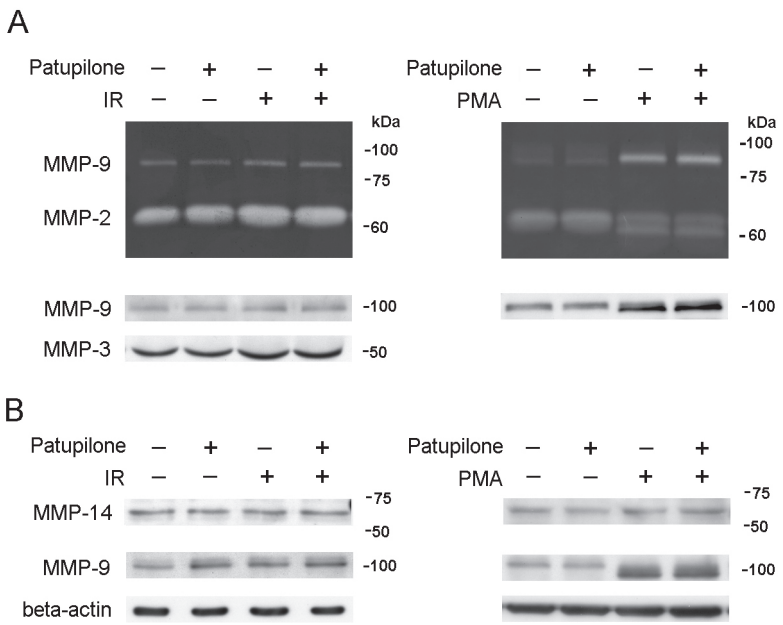

Figure 3.16. Protein level of MMPs is not affected by patupilone treatment. The MMP protein levels in HT1080 cells were determined (A) in the CM by Western blot and by gelatine zymography and (B) in the whole cell lysates by Western blot. The cells were treated with 0.2 nM patupilone 24 h before 10 Gy IR or application of 40 µg/ml PMA. 20 hours thereafter, the cell lysates and the CM were collected. N≥4.

and the MMP-3 at 50 kDa (predicted size 45 kDa). We expected to observe both zymogen and an active form of MMP-14 in the whole cell lysate. However, only one strong band was detected at a size of approximately 60 kDa (predicted sizes are 66 kDa for MMP-14 zymogen and 57 kDa for active form), accompanied by multiple minor bands of various sizes, which were probably unspecific.

The treatment of cells with 10 Gy IR slightly induced the protein level of all tested MMPs in CM, as it was determined by western blotting and zymography assay (Fig. 3.16A, left). Patupilone treatment altered neither the basal protein level nor IR-induced protein level of MMP-2, -3 and -9 in CM. In whole cell lysates, we observed a slight increase in MMP-9 protein levels after all of the applied treatments. However, the MMP-14 protein level did not change in whole cell lysate after any treatment applied. We also assessed amount of the MMP proteins after treatment with PMA and patupilone. As expected from the increased MMP-9 transcription after PMA administration, the MMP-9 protein level was upregulated both in the cell lysate and in the CM, (Fig. 3.16, right). MMP-2 protein levels were not changed after indicated treatments, as can be seen on the zymography gel. Interestingly, in CM from PMA treated cells a double-band of MMP-2 active form can be observed, which probably represents truncated active forms of the proteinase. We also found a slightly up-regulated MMP-14 protein level, even though we did not observe an increase on the level of mRNA (Fig. 3.14A). The pre-treatment with patupilone neither altered the basal level of MMP nor reduced the protein levels that were up-regulated after PMA treatment.

These findings indicate that the inhibitory effect of patupilone on the IR-induced MMP activity was not due to down-regulation of MMP protein expression or secretion, as treatment with patupilone did not significantly affect extracellular and intracellular protein levels of MMPs in HT1080 cells.

3.3.5 Patupilone down-regulates extracellular protein levels of TIMP-1 and TIMP-2

No significant changes of MMP mRNA or protein levels after treatment with patupilone were observed. Thus patupilone-dependent regulation of MMP activity is not due to the influence of the compound on MMP protein expression. Tissue inhibitors of MMPs (TIMPs) are important regulators of MMP activity. On the one hand, TIMPs are potent natural inhibitors of MMP enzymatic activity, on the other hand, TIMP-2 was shown to be a co-factor for extracellular MMP-14-mediated activation of the MMP-2 zymogen (see Chapter 1.4.4). The counteracting action of patupilone on the IR-dependent induction of MMP activity may be a result of patupilone interference with regulatory functions of TIMPs. Therefore, we investigated the effect of IR and patupilone on TIMP-1 and -2 transcription and protein expression.

On the level of transcription, we could only observe a minimal change of mRNA levels in response to irradiation. TIMP-1 and TIMP-2 mRNA levels increased less than 1.5-fold even after irradiation with 10 Gy (Fig. 3.17A). The basal and IR-induced TIMP mRNA levels were not affected by pre-treatment with patupilone. Treatment of cells with PMA induced the transcription of TIMP-1 (up to 2-fold, P=0.045) but not of TIMP-2 [186] (Fig. 3.17B). Again, patupilone did not influence PMA-regulated TIMP-1 transcription.

The protein levels of TIMP-1 and TIMP-2 were assessed by Western blotting in CM and cell lysates after treatment with patupilone and IR or PMA. IR increased protein levels of both TIMP-1 and TIMP-2 in cell lysate and CM, compared to control cells (Fig. 3.18A, B, left). Protein levels of TIMP-1 and TIMP-2 in cell lysates after patupilone treatment were slightly elevated, when compared to control cells. We did not detect any changes in the basal level of TIMP proteins in CM after treatment with patupilone. The protein level of secreted TIMP-2 after treatment with IR in combination with patupilone was reduced, when compared to the protein level in CM after treatment with IR alone (P=0.02). For TIMP-1 the same trend could be observed but the difference was less pronounced. Contrary to the effect

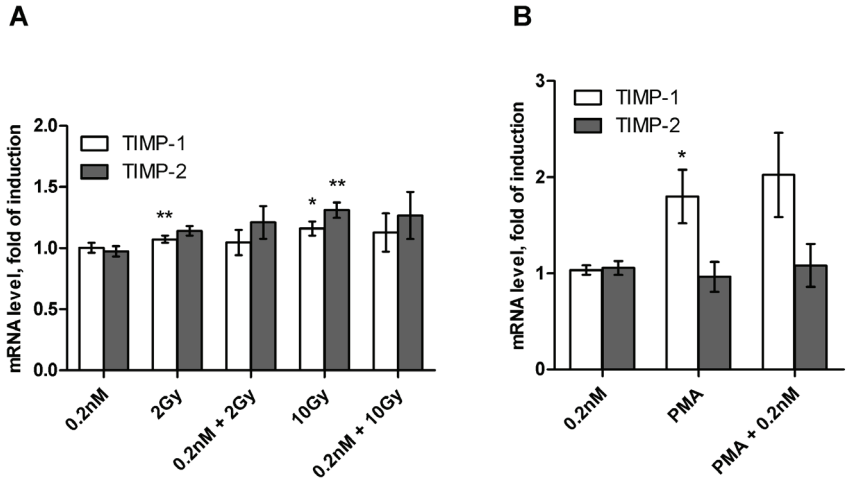

Figure 3.17. mRNA levels of TIMP-1 and TMP-2 are unaffected by patupilone treatment. HT1080 cells were treated with patupilone 24 h prior to irradiation (A) or application of 40 µg/mL PMA (B). 20 h thereafter, mRNA was isolated for the qRT-PCR analysis. Data is shown as mean +/- SE, n≥4, *P<0.05, **P<0.01.

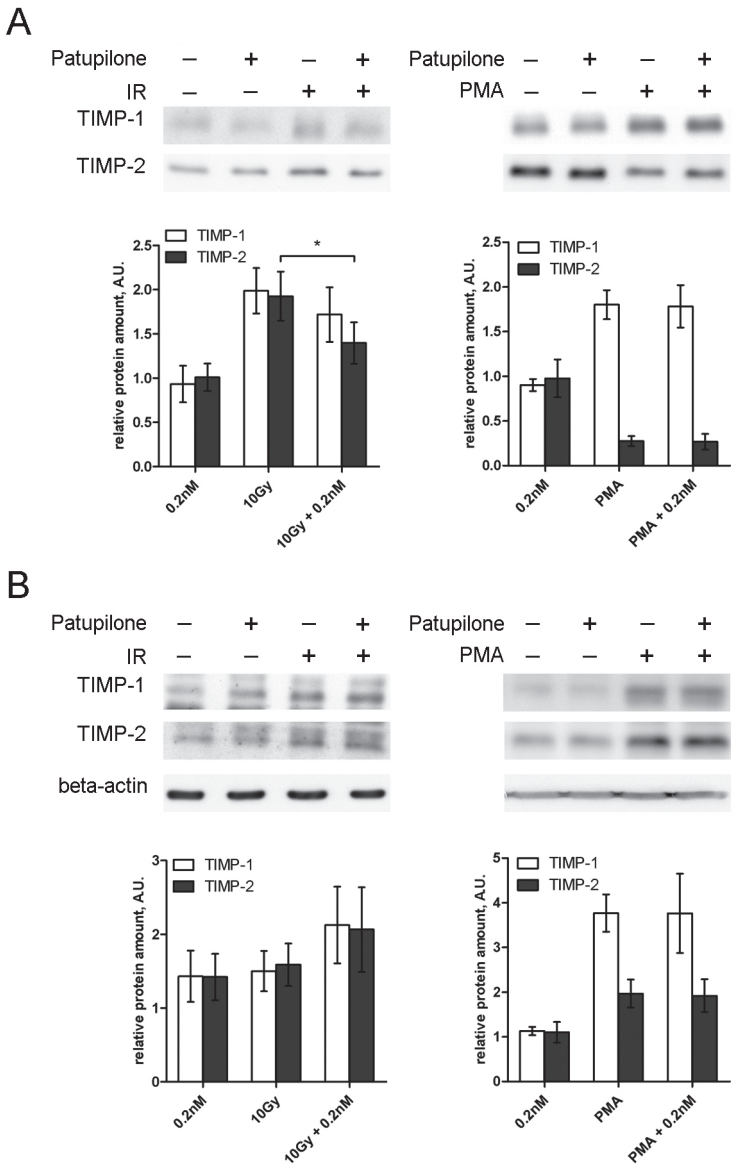

Figure 3.18. IR-induced protein levels of the secreted TIMP-1 and TIMP-2 are reduced after patupilone treatment. HT1080 cells were treated with patupilone 24 h prior to irradiation or application of 40 µg/mL PMA. The protein levels of TIMPs in the CM (A) or in the cell lysates (B) was determined by Western blot 20 h thereafter. The representative blots are shown; quantification was made for at least 4 independent experiments, presented as mean +/- SE.

observed in CM, TIMP protein levels in cell lysates after combined treatment with IR and patupilone were increased, when compared to IR treatment alone (Fig. 3.18B). The TIMP protein accumulation in cell lysate after patupilone treatment with concurrent reduction of the protein level in extracellular space indicates that TIMP protein secretion might be inhibited by patupilone.

Exposure of the cells to PMA increased the amount of TIMP-1 protein both in the cell lysate and in CM. TIMP-2 protein levels were also up-regulated in the cell lysate, however, the level of TIMP-2 protein in CM was drastically reduced (Fig. 3.18A, B, right). Thus the accumulation of TIMP-2 protein in the cell lysate after incubation with PMA may result from reduced secretion. Pre-treatment with patupilone did not affect PMA-regulated TIMP-2 or TIMP-1 protein levels.

These results demonstrate that treatment with patupilone does not affect TIMP-1 and

TIMP-2 transcription (either basal or induced). However, the amount of TIMP proteins in CM is reduced after combined treatment with IR and patupilone, in comparison to radiation treatment alone.

3.3.6 TIMP proteins are required for MMP activity induction after irradiation

While IR increased TIMP protein levels, patupilone treatment reduced the IR-upregulated TIMP protein levels in the CM. Thus TIMP-1 and TIMP-2 may play a role in the mechanism of down-regulation of the IR-induced MMP activity by patupilone. To investigate whether TIMPs are involved in the aforementioned mechanism, we interfered with TIMP protein levels in one of two ways: by using a siRNA approach or with a TIMP protein function using TIMP-neutralizing antibodies (nABs).

MMP activity was assessed in the CM of cells treated with 10 Gy IR and/or 0.2 nM patupilone after down-regulation of TIMP-1 and TIMP-2 proteins using siRNA. The cellular response to treatment in control (siLuc)-transfected cells was the same as in non-transfected cells. MMP activity in the CM was increased after treatment with IR, while pre-treatment with patupilone significantly diminished the IR-induced MMP activity in the CM (relative increase was 1.9±0.13 and 1.6±0.14 after irradiation alone and combined treatment, respectively, P=0.0002, Fig. 3.20A). The transfection of cells with siTIMP-1 and siTIMP-2 led to a down-regulation of TIMP protein levels which was maintained for the entire duration of the experiment (Fig. 3.19). In the CM derived from TIMP depleted cells, IR-dependent induction of MMP activity was weak and there was no longer any observable difference between MMP activity in the CM after treatment with IR alone and in combination with patupilone. In response to both treatments only a 1.5-fold increase in MMP activity could be

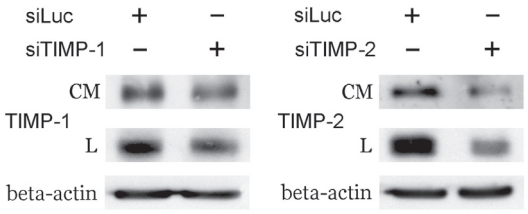

Figure 3.19. Down-regulation of TIMP protein levels by siRNA. The effectiveness of protein down-regulation in HT1080 cells was assessed by Western blot 96 h after transfection with siTIMP-1 or siTIMP-2 in the cell lysate and in the cell culture media supernatant. siLuc was used as an irrelevant control siRNA.

determined (Fig. 3.20A), which was in the same range as the level of MMP activity in non-transfected cells after combined treatment (Fig. 3.4).

To confirm the result obtained with the siRNA-based approach, and in an attempt to assign the observed effect to the secreted TIMPs, we performed the experiment with cells that were exposed to neutralizing ABs against TIMP-1 or TIMP-2 during radiation and patupilone treatment. With control antibodies (goat IgG), as well as with the anti-TIMP-1 neutralizing ABs, the increase of MMP activity in CM was 2-fold after treatment with IR alone and 1.5-fold (P=0.0047) after combined treatment with IR and patupilone (Fig. 3.20B). These treatment-dependent changes were also observed in the CM of cells without AB administration

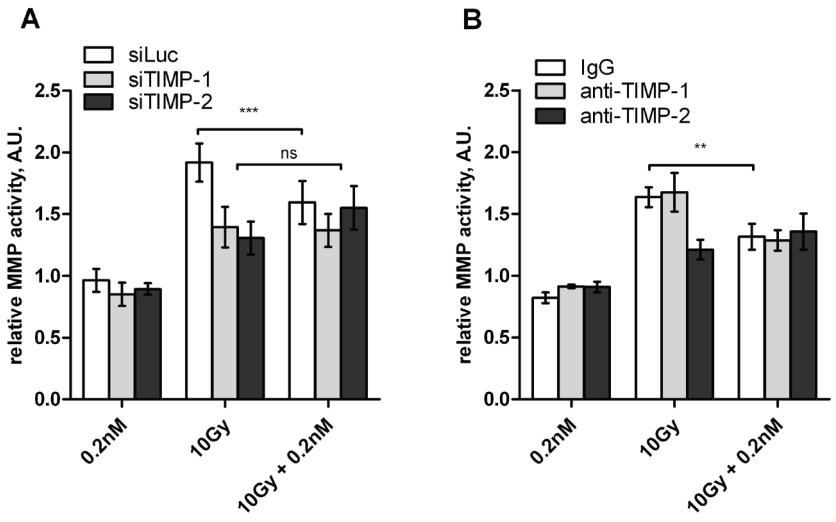

Figure 3.20. Down-regulation of the TIMP protein levels prevents the IR-mediated MMP activity induction. The MMP activity was determined in the CM of HT1080 cells after treatment with patupilone 24 h prior to IR. Mean +/- SE, n≥4, *P<0.05, **P<0.01, ***P<0.001. (A) Anti-TIMP-1, anti-TIMP-2 or isotype control antibodies were applied on the cells at a concentration 0.7 µg/mL thrice: simultaneously with patupilone, 1 h before irradiation and with the serum-free media. (B) TIMP-1 or TIMP-2 was down-regulated using the siRNA approach. The cells were transfected with 200 pmole of siTIMP-1 or 100 pmole of siTIMP-2 and siLuc.

(Fig. 3.4). However, treatment with anti-TIMP-2 neutralizing ABs resulted in an only 1.5-fold increase of MMP activity after IR (P=0.0073). Thus cell exposure to anti-TIMP-2 neutralizing ABs had the same effect on the treatment response on the level of MMP activity as depletion of TIMP-1 or -2 protein levels with the siRNA approach. This data suggests that TIMP-2, but not TIMP-1, is required for the up-regulation of MMP activity after IR treatment. This contradicted the results obtained in the experiment with siRNA directed against TIMP-1 and TIMP-2, which demonstrated the equal importance of both TIMPs. The contradiction could occur due to technical limitations. According to the manufacturer's description, anti-TIMP-1 neutralizing antibodies are less potent than anti-TIMP-2 neutralizing antibodies. Molar ratio TIMP:AB is recommended 1:3 and 1:5 for 50 % neutralization efficiency for anti-TIMP-2 and anti-TIMP-1, respectively.

Overall, the data indicates that the depletion of TIMP-1 or TIMP-2 prevents the activation of secreted MMPs after IR and suggests that patupilone counteracts IR-upregulated MMP activity through the regulation of TIMP-1 and -2 protein levels in CM.

3.3.7 Counteracting effect of patupilone to IR-dependent induction of MMP activity is due to interference with the MMP activation cascade

TIMP-2 is a co-factor of the proMMP-2 activation in a well-described MMP-14 (MT1-MMP)-dependent activation mechanism. The same role is proposed for TIMP-1 in proMMP-9 activation [120]. Our results demonstrated that patupilone reduced the amount of secreted TIMP proteins in CM after radiation (Fig. 3.18) and that TIMPs are required for the IR-dependent MMP activity upregulation, since after TIMP depletion with siRNA or neutralizing antibodies, the level of IR-increased MMP activity did not exceed the MMP activity level after combined

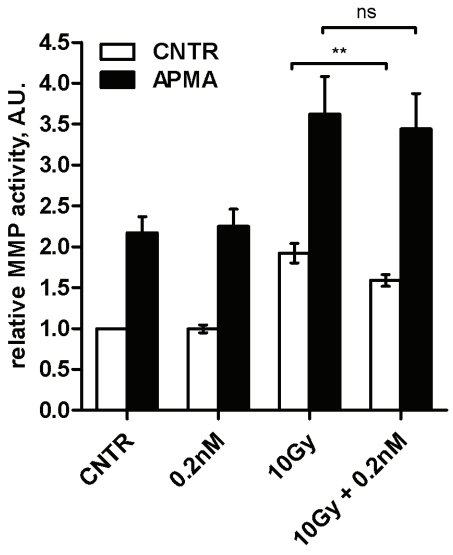

Figure 3.21. Patupilione counteracts the IR-induced MMP activity by an interference with the MMP activation. The MMP activity was determined in The CM of HT1080 cells treated as on Fig. 3.4 with the indicated doses of patupilone and IR. The CM was incubated with 1 mM of APMA for 1 h at 37 °C for MMP activation or control buffer. Mean +/- SE, n=4, **P<0.01.

treatment (Fig. 3.20). Taken together, all this suggests that patupilone may interfere with the process of MMP activation after irradiation by depleting secreted TIMPs. To further test this hypothesis, we used the universal, unspecific activator of MMPs 4-amino-phenylmercuric acetate (APMA), which activates MMP zymogens by inducing conformational change independently of natural regulation cascades (other MMPs and TIMPs) [187].

The CM samples of HT1080 cells, tested previously in the MMP activity assay (Fig. 3.4), were incubated with APMA (1 mM) for 1 h at 37 ºC and the MMP activity was measured once more. After activation with APMA, the measured MMP activity levels approximately doubled for all treatment conditions. In the APMA-activated samples, the MMP activity was 1.7-fold higher in the CM of irradiated cells as compared to control cells. However, we could not observe the counteracting effect of patupilone on IR-induced MMP activity. The MMP activity levels in CM from cells treated with IR alone or in combination with patupilone were similar in APMA-activated CM (Fig. 3.21). This result confirmed that patupilone down-regulated IR-induced MMP activity due to interference with proMMP activation processes, as the inhibitory effect of patupilone could no longer be observed when proMMPs in the CM were forced to an active state.

In an attempt to uncover mechanisms of the patupilone counteracting effect to the IR-dependent induction of MMP activity, we dissected the MMP regulation cascade on different levels. Patupilone did not influence IR-induced events on the level of cell cycle distribution and transcription. Namely, it did not interfere with accumulation of cells in G2/M phase after IR (Fig. 3.11B) and did not alter the IR-induced transcription of MMPs (Fig. 3.13) and TIMPs (Fig. 3.17A). Both MMP and TIMP protein levels were elevated in cell lysate and CM after treatment with 10 Gy of IR. Patupilone did not influence intracellular and extracellular MMPs protein levels (Fig. 3.16).

However, the amount of extracellular TIMP-1 and TIMP-2 proteins after combined treatment with patupilone and IR was lower than after treatment with IR alone (Fig. 3.18A). Furthermore, the counteracting effect of patupilone on IR-induced MMP activity was not observed in CM of TIMP-1 and TIMP-2 depleted cells (Fig. 3.20). After TIMP depletion, the level of IR-increased MMP activity did not exceed the MMP activity level after combined treatment, indicating that the proMMP activation processes after treatment with IR requires TIMPs. These results strongly suggest that patupilone counteracted IR-induced MMP activity due to interference with the MMP activation processes by depleting the levels of secreted TIMP-1 and TIMP-2. This proposition was further confirmed by the finding that the inhibitory effect of patupilone on the IR-induced MMP activity could not be observed in CM after ex vivo activation of pro-MMPs (with APMA, Fig. 3.21).

4 Discussion

4.1 Radiosensitizing properties of patupilone

Microtubule stabilizing agents (MTSAs) have been tested in combination with ionizing radiation (IR) on the preclinical level in the *in vitro* and *in vivo* setting, revealing radioenhancement ranging from the additive to the supra-additive dimension. In the present work we confirmed the radiosensitizing effect of patupilone in a wide range of human cancer cells (colon carcinoma, fibrosarcoma, glioma and medulloblastoma). Patupilone already revealed its radiosensitizing capacity at very low concentrations, which were not cytotoxic, when applied alone and did not alter the microtubular (MT) structure of the cells. No alterations in cell cycle distribution were observed after treatment with these effective patupilone doses. One of the possible mechanisms of action at such a low concentrations may be reduction of MT dynamics with consequent aberrant mitosis and loss of replicative potential (clonogenicity) of the tumor cells [77-78]. There are also indications that DNA repair might be influenced by MTSAs. The tumor suppressor p53 is found to localize with cellular microtubules and treatment with MT-stabilizer paclitaxel and destabilizer vincristine reduced the nuclear accumulation of p53 and the expression of mdm2 and p21 in response to DNA-damage [188]. When A549 cells were treated with low concentrations of MT-interfering drugs (which did not induce either mitotic arrest or change in tubulin polymerization rate) prior to doxorubicine exposure, nuclear accumulation of p53 was enhanced. This resulted in apoptotic cell death and sensitisation to doxorubicine [189].

MTSAs as chemotherapeutics have been in clinical use already for some decades [55-58] but the step towards bringing combined treatment with MTSAs and IR into clinics is still in its initial phase. Phase I and II clinical trials demonstrated that combined treatment with paclitaxel and IR is well tolerated by patients with a broad range of cancers [190-194]. A recent Phase I study showed that patupilone can be safely combined with RT for malignancies of the central nervous system [195]. Even though the radioenhancing properties of MTSAs are widely accepted by the research and clinical community, surprisingly little is known about the mechanisms of their radiosensitizing effect. Our recent studies with patupilone challenged the long-standing dogma of the M phase arrest-related mechanism of radiosensitization ([77, 79]. Our data also confirmed the accumulation of cells in the S phase after patupilone treatment in human colon carcinoma and fibrosarcoma cells, which was previously reported in

MDR-overexpressing colon carcinoma cells [79]. Further research is still required to determine whether or not S phase accumulation is involved in a radiosensitizing effect, and if so, how this occurs.

The previous research work of our colleagues indicated a tumor microenvironment-oriented mode of action of the combined treatment with patupilone and IR [79, 157]. In the present study, we elucidated the effect of patupilone on IR-induced processes in the cancer cell microenvironment, with a focus on the MMP function.

4.2 Effect of combined treatment with patupilone and IR on MMP function

MMPs play an important role in cancer progression by remodelling the extracellular matrix and regulating multiple biologically active molecules (reviewed in [132, 139, 179]. Based on their substrate profile, both the "cancer-promoting" and "cancer-inhibiting" functions of MMPs were proposed. However, overall, the sum of MMP actions promotes cancer progression (see Chapter 1.4.5). This assumption is supported by a great body of data showing that MMPs are overexpressed in multiple tumor types and correlate with aggressiveness of the disease (reviewed in [139]). Radiation treatment up-regulated MMPs in pre-clinical studies [32-33] and in patients with lung and breast cancer after radiotherapy [172]. MMPs are secreted both from tumor cells and cancer stromal cells. Studies of Bley and co-workers [157] and Escuin and co-workers [166] demonstrated that tumor cells mediate the influence of MTSAs on the tumor microenvironment. Thus, treatment with MTSAs did not result in disruption of tumor vasculature or changes in hypoxia levels or HIF-1 transcriptome in tumors, which originated from drug-resistant tumor cell lines, in contrast to their drug-sensitive counterpart tumors. These findings indicate that close investigation of tumor cell-secreted factors upon treatment with MTSA and IR may cast light on mechanisms of radioenhancement.

The vast majority of the known functions of MMPs are related to their enzymatic activity [139]. As such, we investigated the effect of clinically relevant treatment modalities, namely ionizing radiation and the chemotherapeutic agent patupilone, on the activity of MMPs. In particular, we investigated the activity of MMPs that are secreted from human cancer cells in response to the indicated treatment. Our main experimental system was the HT1080 human fibrosarcoma cell line which is a very well established model for investigation of MMP function and cell invasion [178, 196-198]. Another cell line that was used for the experiments was U251 cells, which originate from a human glioma, highly invasive and treatment-resistant tumor type [199-201].

We demonstrated that patupilone at low concentrations significantly decreased the IR-induced MMP activity in conditioned cell culture media (CM) of these two different cancer cell lines. Patupilone also reduced the basal level of MMP activity in CM. At these effective

concentrations, patupilone had a minimal cytotoxic effect but exhibited radiosensitizing properties in the cell survival clonogenic assay. There is evidence that not only secreted MMPs but also MMPs at the cell surface, so-called MT-MMPs, are highly relevant to cell invasion, as they can activate proMMPs and, additionally, degrade components of the ECM by themselves [202-204]. Therefore, we checked whether the cell membrane-associated MMP activity was influenced by treatment with IR and/or patupilone but we did not observe any significant influence. This indicated that in our system the major effect of treatment with IR and patupilone was on the secreted MMP activity.

In the present work, we assessed MMP activity in CM using a FRET substrate-based assay that allows to determine the MMP activity in the native supernatant [205-206] without any specific pre-processing. The only drawback of the assay is that it is not possible to selectively determine the enzymatic activities of individual MMPs. Selective substrate peptides and inhibitors for individual MMPs are not yet available because of the great similarity of the catalytic centres of the MMP family members [94]. Studies in the MMP field very often imply gelatin zymography [33, 100, 102] for the selective determination of MMP activity in body fluids or cell culture media. However, this technique actually measures an amount of MMP proteins, both active forms and zymogens, without considering the complex formation of MMPs with other regulatory molecules. This is a result of a technical limitation: complexes are denaturated during separation in an SDS polyacrylamide gel. In addition, MMP zymogens are non-proteolytically activated by conformational changes in the presence of SDS [185, 207]. Thus the results of zymography assay rather reflect the MMP protein expression levels than the MMP activity *in vivo*. A direct correlation of MMP expression to function is not possible because of complex MMP activation and regulation processes [108]. The secreted MMP zymogens are activated in the extracellular space by mechanisms that have yet to be fully identified. The activated proteins may form complexes with TIMPs, which leads to the inhibition of enzymatic activity. Due to this complex regulatory cascade, only a certain fraction of expressed MMPs exhibit proteolytic activity in the extracellular space and the size of this fraction is difficult to predict. We believe that direct determination of MMP activity in conditioned media – in contrast to assessment of the MMP expression – is the most relevant experimental endpoint, as MMP activity translates directly in most of MMP biological functions [132]. For this purpose, the substrate cleavage assays (such as the FRET substrate-based assay described above) that allow to determine the MMP enzymatic activity directly in biological samples are more accurate than the zymography assay.

To confirm that the increase of MMP activity after IR and patupilone down-regulation thereof is relevant to the functional cellular level, we assessed cell invasion after the respective treatments in a transwell chamber assay. This model system mimics the first step of metastasis: invasion of the tumor cell through the basal membrane. The cell invasion does not reflect the complexity of all MMP functions, as the MMP substrate repertoire is much

wider than the MMP-substrate components of the ECM. However, the transmigration assay is highly specific for MMP function as MMPs are required for the ECM degradation and successful transmigration [180]. The results of the cell invasion experiments with the human fibrosarcoma and glioma cells in the present study were well correlated with the results of the MMP activity experiments. The cell invasion of both human fibrosarcoma and glioma cells in the basement membrane mimicking system (Matrigel™) was increased after irradiation. Pre-treatment of cells with patupilone abrogated this IR-induced increase of cell invasiveness. Treatment with patupilone alone decreased the basal level of cell invasiveness (in fibrosarcoma cells). Cell invasion requires two functional components: cell motility and the degradation of extracellular matrix. Patupilone at low concentrations did not affect cell motility. Thus, the inhibitory effect of patupilone on cell invasion has to be attributed solely to a decrease of ECM-degrading capacity after treatment. The increase of cell invasion after IR was observed only at a low dose (up to 2 Gy). Higher doses of radiation led to the opposite effect – cell invasion was inhibited. This differential effect of IR on cell invasion is most likely related to IR-induced cytotoxicity. Cell invasion and motility in our experiments were decreased with IR dose escalation in correlation with IR-induced cytotoxicity (especially in glioma cells). Other reports previously demonstrated that only sub-lethal doses of IR induce cell invasion, which is inhibited by increasing cytotoxic IR doses [35, 208]. This phenomenon can explain the controversy in the literature on this topic. Both induction and suppression of cell invasion after IR were reported and it is difficult to draw a conclusion as the effect on cell survival at the corresponding doses of IR was not always described [28-31, 34]. The induction of cell invasion by sub-lethal radiation doses is a particularly important phenomenon in context of fractionated radiation treatment as the tumor dissemination rate may increase between the fractions.

The increase of cell invasion after IR can be attributed to enhanced MMP activity as a specific MMP inhibitor NNGH could inhibit cell invasion, both of untreated and irradiated cells. Interestingly, in our experiments we did not observe any inhibition of invasion by recombinant TIMP proteins (rTIMP), as shown in other experimental systems [209-210]. In our experiments, rTIMP-1 and -2 also could not inhibit MMP activity in conditioned media. TIMPs have very high affinity to MMPs and rapidly form complexes with the proteases. Furthermore, the complex formation does not always lead to inhibition of the MMP enzymatic activity but can also serve proMMP activation [108, 120, 131, 211]. There is evidence that TIMP-proMMP complexes may be formed inside the cell before secretion. Roderfeld and coworkers [181] demonstrated that proMMP-9-TIMP-1 complex is already present in the Golgi apparatus. We assumed that the pre-formed MMP complexes with endogenous TIMPs might prevent rTIMPs from inhibiting MMP activity in our experiment. Therefore, we used conditioned media of cells that had TIMPs down-regulated by siRNA approach; however, we still did not see any inhibitory effect of rTIMPs on MMP activity. Furthermore, incubation of

exogenous TIMPs with conditioned cell culture media of HT1080 cells reduced their ability for inhibition of recombinant MMPs. There are no known natural inhibitors of TIMPs, even if MMPs can be considered so [120]. However, our data suggests that there is indeed an unknown TIMP-scavenging activity in conditioned media of human fibrosarcoma cells.

IR regulates MMP expression on the transcriptional level [28, 170, 183]. Promoter regions of MMPs contain binding sites for transcriptional factors, which are induced after activation of stress signalling pathways (e.g. MAPK and Nf-kB) [73-74, 96, 212]. This makes MMPs a part of a cellular stress response. Our results strongly supports this assumption. The treatment of cells with hydroxyurea or aphidicolin and the subsequent S phase arrest led to an elevation of MMP activity in CM and up-regulation of MMP expression on a transcriptional level. We also observed a strong increase of MMP activity in CM after treatment with high cytotoxic doses of microtubule-interfering agents (patupilone, nocodazole, vincristine) and doxorubicine (data not shown). The mechanism of MMP activation after the above-mentioned stimuli is also most probably related to the activation of the stress response pathways. Doxorubicine was shown to activate MMP-9 expression via p38MAPK and MMP-2 expression via ERK and JUNK signalling cascades [212]. Paclitaxel induced the Nf-kB pathway in breast and ovarian cancer cells [73-74]; patupilone also induced apoptosis in colon carcinoma cells via activation of Nf-kB pathway [75]. Since MMPs can activate growth and survival factors [115] and may be involved in the protection of cells from apoptosis [116], they indeed can be an important component of stress response.

Data on the transcriptional effects of patupilone and paclitaxel is rather poor. Treatment with high cytotoxic concentrations of the compounds leads to the activation of the aforementioned stress response pathways [73, 213] and to the induction of cell cycle checkpoint-related genes. This is most likely a result of MT stabilization and MTSA-induced M phase arrest [71]. No alterations in the gene expression were reported for low concentrations of MTSAs and we also could not observe any effect of patupilone at a low concentration on gene expression of MMPs and TIMPs.

MMP activity may be regulated by MTSAs on the level of MMP protein secretion. The intracellular transport of MMPs and TIMPs depends at least in part on an intact MT system [113-114, 174]. Paclitaxel treatment impaired the secretion of MMP-2 and -9 and significantly reduced invasive activity of melanoma cells [48]. In our attempt to elucidate the mechanism of the counteracting effect of patupilone on IR-induced MMP activity, we excluded cell cycle-related and transcriptional mechanisms, as patupilone did not interfere with IR-induced G2 arrest and IR-induced MMP transcription. Our assumption that patupilone mediates its counteracting effect on IR-induced MMP activity via microtubule- and secretion-related mechanisms is supported by the following findings: IR-induced MMP activity was also down-regulated after treatment with other microtubule-interfering agents, such as paclitaxel and nocodazole. Furthermore, we observed an accumulation of TIMP proteins inside the cells and a

reduced amount of TIMP protein levels in the CM after combined treatment, when compared to radiation treatment alone. However, the intra- and extracellular levels of MMP proteins did not change after treatment with patupilone. This indicated that the secretion of TIMPs, but not MMPs, could be inhibited by MTSA. Various mechanisms of intracellular transport of different MMPs and TIMPs were described previously. Sbai et al [114] demonstrated that, in mouse neuronal cells, the co-localization of overexpressed MMP-2, MMP-9 and TIMP-1 proteins with MT-dependent molecular motor kinesin differs substantially. TIMP-1 was associated with kinesin to a lower extent than MMP-2 and MMP-9. This study does not directly explain our findings. However, it strongly indicates that secretory mechanisms may play a role in the specific regulation of individual MMPs and TIMPs levels and activity, and that we can only start to understand these processes at the molecular level.

We demonstrated that IR-induced TIMP-1 and TIMP-2 extracellular protein levels were diminished by patupilone. This finding indicated that TIMPs might play a role in the inhibitory effect of patupilone on IR-induced MMP activity. Indeed, in the present study, IR failed to enhance MMP activity to the full extent if cellular and secreted TIMP-1 or TIMP-2 protein levels were down-regulated. After TIMP depletion, the level of IR-increased MMP activity did not exceed the level of MMP activity after combined treatment, indicating that a) the proMMP activation processes after irradiation require TIMPs, and b) patupilone interferes with the activation process by regulating extracellular TIMP levels. Additionally, the activation of MMPs in CM with 4-aminophenylmercuric acetate (APMA) leads to similar levels of MMP activity in CM, when collected after treatment with IR (both alone and in combination with patupilone). APMA activates MMPs potently and non-specifically by chelating cystein in the pro-enzyme domain. This leads to conformational change, which rescues the catalytic center. Therefore, an increase of MMP activity after incubation with APMA is due to the activation of MMP zymogens, which are present in CM, while the activity of MMPs that are trapped in inhibitory complexes with TIMPs remains concealed [106]. The equal activity in APMA-treated CM of cells after radiation and combined treatment also reflects the equal MMP protein levels after these treatments.

Taken collectively, our results suggest that the counteracting effect of patupilone on IR-induced MMP activity can be attributed to a reduction of MMP zymogen activation processes after patupilone treatment due to depletion of secreted TIMPs (see Outlook, chapter 5.1), which play a role in proMMP activation. In our study, we showed that both TIMP-2 and TIMP-1 are important for the induction of MMP activity after radiation treatment. This is an important finding, since the role of TIMP-2 in activation of proMMP-2 is well known, but the role of TIMP-1 in proMMP-9 activation has only been predicted based on the structural similarities of their complexes [120]. It is widely appreciated that the ratio of TIMPs and MMPs is a very important factor in the regulation of MMP activity. According to the current model of proMMP-2 activation by MT1-MMP [108], TIMP-2 is forming a complex with

proMMP-2 and serves as an adaptor for binding to MT1-MMP. A certain level of TIMPs is required for optimal activation [131] and both reduction and increase of TIMP-2/proMMP-2 ratio leads to inhibition of proMMP activation either due to a lack of "adaptor" TIMP-2 or as a result of a competition between "empty" TIMP-2 and TIMP-2/proMMP-2 complexes for MT1-MMP binding site [108, 211]. In addition, TIMP-2 can inhibit proteolytic activity of the activated MMP-2. The present work confirmed that the TIMP/MMP ratio is important for proMMP activation. More importantly, we demonstrate that patupilone disturbs this ratio and thereby interferes with MMP activation after IR.

4.3 Translational significance

To the best of our knowledge, we have demonstrated, for the first time, that patupilone can counteract IR-induced activity of secreted MMPs and subsequent increase of cell invasion. Of particular interest is that the counteracting effect of patupilone can be observed in human glioma cells. Glioblastoma (or grade IV glioma) is one of the most treatment-resistant primary tumors in adults. Despite of recent introduction of the new chemotherapeutic agent temozolomide to the standard therapy of glioblastoma, median overall survival still remains at a rate of 14 months. Thus, the challenge remains to improve the treatment outcome [200]. The main established reasons associated with poor prognosis are extensive infiltration of surrounding brain tissue by tumor cells and the blood-brain barrier as an obstacle for the adequate delivery of cytotoxic agents [214]. Radiation therapy is a standard treatment for gliomas in spite of evidence that irradiation can promote glioma cell migration and invasiveness in vitro and in vivo, which can be mediated by up-regulation of MMPs [201, 208, 215]. Patupilone was already shown to decrease the occurrence of lymph node metastasis in mouse orthotopic prostate tumor model. [91]. The ability of patupilone to cross a blood-brain barrier and to retain in brain tissues [216-217] makes patupilone a very potent and promising chemotherapeutical agent for brain malignancies. The capacity of patupilone to inhibit IR-induced glioma cell invasion, shown in the present study, increases the value of the compound as candidate for a combined treatment modality with radiation therapy for glioblastoma treatment.

MMPs were, for a long time, considered to be pro-malignant factors on the basis of their massive upregulation in malignant tissues. The pharmacological industry puts a substantial effort into the development of MMP-inhibitors (MMPIs) as anti-cancer agents. MMPIs were exhibiting good efficacy in pre-clinical studies. For example, batimastat (a broad-spectrum MMP inhibitor) reduced metastasis and inhibited local re-growth of tumors in nude mice after resection of human breast cancer xenografts [218]. MMPIs for cancer treatment reached Phase III clinical trials but so far all of them failed to show any clinical benefit for cancer patients and exhibited severe side effects including musculoskeletal pain and inflammation [219-221]. One of the possible reasons for such an unsuccessful entry in the clinic was the

unspecific nature of these inhibitors. Regulation, structure and substrates of MMPs overlap to a great extent. Therefore, research on this group of proteins is not a trivial task and there are no inhibitors that are selective to certain MMP family members. Broad-spectrum inhibition was ineffective in patients; however, highly selective and specific inhibition of MMPs could still be an effective treatment for cancer, if care is taken to target only relevant MMPs in specific disease settings.

Clinical trials with MMPIs also met difficulties in interpretation. There is a discrepancy between the design of pre-clinical and clinical studies. While in the animal model studies, MMPIs were administered at early disease stage and maintained throughout disease progression with tumor growth control as an experimental endpoint, human clinical trials focused on administration of inhibitors only at end stage disease. Thus, the best window for MMPI treatment may have been missed [220, 222]. Furthermore, MMPIs do not have antiproliferative properties and are therefore not expected to result in responses assessed by classical clinical endpoints for cytotoxic agents. Instead, stable disease must be accepted as the best response, at least in case of MMPIs, as a single treatment modality [219].

An additional possible reason for the failure of MMPIs is the protective role of MMPs in tumor progression that has been recognized only during the last decade. Most of the data derives from genetic studies in murine models of tumor progression where ablation of specific MMPs actually resulted in accelerated disease progression. For example, squamous cell carcinomas that developed in MMP-3-knock-out animals had a faster initial rate of growth and tumor progression as compared to their wild-type controls [223]. HPV16-driven carcinomas that arose in MMP-9 deficient mice were more aggressive and of higher grade, compared to tumors found growing in wild-type animals. The authors determined that the MMP-9 effect derived from stromal inflammatory cells [224]. MMP-9 activity is also important for the generation of angiostatin and tumstatin (anti-angiogenic factors), as was shown in a study where large angiogenic tumors occurred in MMP-9-knock-out mice [225]. Finally, all MMPIs tested to date were directed against the proteolytic activity of MMPs. However, MMPs also reveal non-proteolytic functions that are mediated via their hemopexin domain. The hemopexin domain of proMMP-9, but not its proteolytic activity, was necessary for MMP-9-mediated epithelial cell migration in a transwell chamber assay [226]. The cytoplasmic tail of MMP-14 carries a migration-promoting function on macrophages [227]. In chronic lymphocytic leukemia, MMP-9 promotes B cell survival in a non-proteolytic fashion via its hemopexin domain by induction of the intracellular signalling, which prevents B cell apoptosis [228]. The nonproteolytic functions of MMPs could also explain why previous clinical trials using inhibitors of the MMP catalytic domains failed as anticancer therapeutics. In spite of all complications, MMPs are a very attractive target and many clinical trials are ongoing with new generation of more selective MMPI compounds [220].

The natural MMP inhibitors TIMPs could also be used for anti-cancer therapy as they

exhibited anti-cancer activity. Ectopic expression of human TIMP-1 in mice inhibited growth of Ehrlich tumor allografts by suppression of neovascularization [229] and brain metastasis of fibrosarcoma xenografts [230]. After being injected into the mammary fat pads of nude mice, TIMP-2 producing tumor breast cells grew slower as xenografts and showed a dramatic decrease in size and number of lung metastatic tumors [231-232]. The adenoviral expression of TIMP-3 in human melanoma xenografts promoted apoptosis in melanoma cells through stabilization of a number of death receptors and activation of their apoptotic signaling cascade through caspase-8 [233]. It was also reported that TIMP-3 deficiency in the host stroma, but not in tumor xenograft, leads to enhanced tumor growth and angiogenesis. Thus, TIMP-3 located within the tumor microenvironment can inhibit tumorigenesis [234].

However, TIMPs can not only inhibit, but also promote, tumor progression through various mechanisms. The expression of TIMP-1 enhanced the tumorigenicity in K14-HPV16 transgenic mice by potentiating keratinocyte hyperproliferation and the appearance of chromosomal aberrations in premalignant cells [235]. TIMP-1 also protected MCF-7 breast cancer cells from paclitaxel-induced apoptosis by decreasing the stability of cyclin B1 [236]. A highly invasive subclone of U87 cells exhibited two-fold elevation of TIMP-2 and over-expression TIMP-2 in parental U87 cells promoted MMP-2 activation and invasion in these glioblastoma cells [237]. TIMPs can activate pro-survival cellular signaling pathways as reported for TIMP-2, which displays growth stimulatory activity mediated by the activation of Nf-kB in A549 lung epithelial cells [238] and for TIMP-1, which enhanced cell growth by inhibition of metalloproteinases and activation of ERK1/2 and p38 [239]. TIMP-1 also plays various roles during tumor development. Early subcutaneous growth and angiogenesis of engrafted B16 melanoma was promoted in mice by overexpressing human TIMP-1 in the liver; however, the lung colonizing ability of the melanoma cells later during tumor development dramatically decreased [240]. Since TIMPs display paradoxical effects on cell proliferation and cell death, it is difficult to generalize their effect on tumor growth and predict the outcome of TIMP interference. The present study demonstrated that the induction of TIMP-1 and TIMP-2 after IR can lead to increased MMP activation and subsequent cell invasion.

Therapeutic agents that cleverly target members of MMP and TIMP families in selective and disease/function-specific ways can represent an interesting class of agents for a combination with ionizing radiation. They can join a group of potential radioenhancing compounds together with DNA repair-interfering agents, signal transduction pathway inhibitors and MTSAs. This study demonstrated that the MTSA patupilone is a compound of great interest. It is a potent inhibitor of tumor cell proliferation under hypoxic and normoxic conditions, a very promising anti-metastatic and anti-angiogenic agent, an inducer of apoptosis, and a potent radiosensitizer in vivo and in vitro as described elsewhere [60, 75, 79, 88, 91-93, 157, 167]. In addition and as demonstrated in the present work, patupilone inhibits IR-induced activity

of secreted MMPs and IR-induced cell invasiveness. Since IR-induced MMP activity may be relevant beyond tumor cell dissemination, due to the complex MMP functions in cancer progression, the combined treatment modality of IR and patupilone might indeed be of a great clinical benefit for cancer treatment.

5 Outlook

5.1 Microtubule dependent secretion of TIMPs protein

The present study demonstrated that patupilone diminishes the IR-induced increase of extracellular MMP activity. This effect of patupilone was observed at a very low dose of the MTSA (e.g. 0.2 nM in HT1080 human fibrosarcoma cell line). This dose was not cytotoxic when applied alone and did not alter the morphology of the microtubular cytoskeleton. However, patupilone at this dose enhanced IR-induced cytotoxicity, exhibiting a radiosensitizing effect. Our findings strongly suggest that the counteracting effect of patupilone on IR-induced MMP activity is attributed to interference with the MMP activation processes due to a depletion of secreted TIMP-1 and TIMP-2 proteins. We demonstrated that after treatment with patupilone, in combination with IR, the amount of secreted TIMPs in conditioned cell culture media of irradiated cells was reduced, when compared to the level of secreted TIMPs after IR treatment alone. We simultaneously observed a trend towards the accumulation of the TIMP proteins in cell lysates. These results indicated that patupilone might interfere with secretion of TIMP-1 and TIMP-2 after radiation treatment. Furthermore, our data suggested that the inhibitory effect of patupilone is linked to the microtubular system, as other MT-interfering agents (paclitaxel and nocodazole) exhibited similar properties and also inhibited IR-induced extracellular MMP activity.

TIMP-1 protein is transported in vesicles, which co-localize with microtubule structures and associated with the MT-dependent molecular motor kinesin, as reported in mouse neuronal cells [114]. MMP-2 and MMP-9 also co-localize with MTs in mouse neuronal cells and in human melanoma cells. Furthermore, secretion of MMPs can be inhibited by the microtubule-stabilizing agent paclitaxel [48, 174]. To our knowledge, we demonstrated for the first time that TIMP secretion may be inhibited by the MTSA patupilone. To confirm this hypothesis, confocal microscopy techniques could be employed. We are currently generating plasmid vectors for expression of fluorescent protein-labelled TIMPs. Figure 5.1 demonstrates an HT1080 cell, which transiently expresses TIMP-1-EGFP: a vesicular subcellular localization of TIMP-1-EGFP can be observed. We intend to generate TIMP-1 and TIMP-2-EGFP expressing cells. These cells will be treated with IR, alone and in combination with patupilone, and the rate of vesicular trafficking will be assessed by Fluorescence Recovery after Photobleaching (FRAP) technique. FRAP allows to measure the fluorescent recovery of a bleached area of

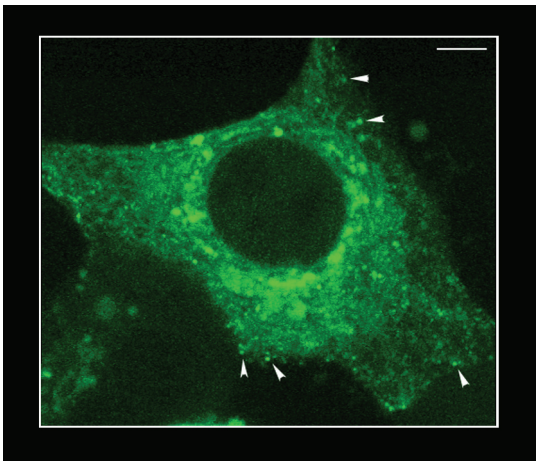

Figure 5.1. Expression of TIMP-1-EGFP in the HT1080 cells. The cells were transiently transfected with the pEGFP-TIMP-1 vector. Arrows indicate the vesicular subcellular localization of TIMP-1-EGFP (green), scale bar 5 µm.

the cytoplasm [241]. We expect a slower fluorescence recovery of a bleached region in cells treated with patupilone, which could indicate impaired MT-dependent vesicular trafficking of fluorescent TIMPs. The drawback of the FRAP approach, however, is that with this technique it is not possible to differentiate between MT-dependent and independent (e.g. cytoplasmic diffusion) vesicular trafficking. To overcome this limitation and to attribute the observed phenomenon to MT-dependent processes, further microscopic observations can be performed. The fluorescent TIMP-overexpressing cells may be stained with life cell fluorescent MT dye (Cellular Lights™, Invitrogen) and the tracking of fluorescent vesicles along the MTs may be performed. Again the rate of movement of TIMP-GFP-labelled vesicles (travelling speed or distance) is expected to be lower in cells treated with patupilone and IR than in those treated with IR alone.

5.2 Clinical aspects of the MMP activity alterations after treatment with patupilone and IR

The present study once again confirmed that IR increases MMP activity and tumor cell invasiveness in a preclinical setting [28-31, 208]. Clinical insights in this area are very limited. We found only two reports in the literature concerning the upregulation of MMP activity in plasma and rectal mucosa of cancer patients after radiotherapy [172, 242]. More intense translational and clinical studies are required to adequately evaluate the role of radiotherapy in the potentially disadvantageous activation of MMPs in irradiated tissues. MMP activity after radiotherapy may be determined not only in tissues obtained by biopsy (invasive method) but also in body liquids, like plasma or urine [33]. Furthermore more intense systemic studies are required to estimate of the role of radiotherapy in tumor dissemination.

We demonstrated herein that patupilone can diminish IR-induced extracellular MMP activity. The biological relevance of this finding can be demonstrated in established human tumor cells, as patupilone also inhibited IR-induced cell invasiveness in vitro. The next step must be a demonstration of the clinical relevance of our findings. Possible experimental

end-points could be: (i) determination of MMP activity in vivo in tissues after combined treatment, (ii) an assessment of tumor invasiveness and tumor cell dissemination in vivo (histological characterization of invasive tumor margins or frequency of distant metastasis) and (iii) histological evaluation of tissue TIMP and/or MMP protein expression after combined treatment with patupilone and IR. As the administration of MTSAs with IR is not a standard treatment modality today, no patient biopsies are available for histological analysis of these endpoints. Currently, the use of animal orthotopic tumor models is the best available approach for addressing these questions.

One of the clinically relevant findings of the present work is that patupilone can inhibit IR-induced invasion of human glioma cells in vitro. Gliomas are very aggressive brain tumors with poor response to currently available therapies [214]. Wild-Bode and co-workers [208] described a rat orthotopic glioma model (L9 cells), with which they could demonstrate that sub-lethal doses of IR induced invasiveness of this brain tumor. We plan to employ this experimental model for an in vivo study with patupilone and IR. Pre-irradiated and highly invasive cells will be injected intracranially and rats will be treated with patupilone. As patupilone can cross the blood-brain barrier [217] and inhibit the IR-induced glioma cell invasion (as shown herein), we expect that brain tumors in patupilone-treated mice should be smaller, fewer satellites and have less invasive margins than tumors in untreated mice. These end-points and expression of TIMP proteins in tumor tissue after combined treatment could be estimated using histological techniques.

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